# THE RELAXANT EFFECT OF NITRIC OXIDE DONOR ON THE CONTRACTILE ACTIVITY OF ILEAL: ROLE OF CGMP AND POTASSIUM CHANNELS

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## ABSTRACT

**Nitric oxide (NO) is a small molecule of endogenous gases and has important physiological functions. It is well known that NO is an inhibitory action of gastrointestinal smooth muscle cells. The aim of this study was to determine the role of cyclic guanosine monophosphate (cGMP) and potassium ($K^+$) channels in relaxation induced by NO of ileal smooth muscles. Sodium nitroprusside (SNP) at doses between ($1X10^{-6}$ to $3X10^{-4}$ M) showed more potent relaxant effect in acetylcholine (ACh) ($10^{-5}$M) than potassium chloride (KCl) (60mM) induced ileal smooth muscle contractions. Before precontraction by ACh, the segments of ileum was incubated for (20) minutes with various blockers such as Methylene blue (MB), Glibenclamide (GLIB), 4-aminopyridine (4-AP), Barium Chloride ($BaCl_2$) and Tetraethylammonium (TEA). In the presence of MB (3mM), 4-AP (1mM), $BaCl_2$ (1mM) and TEA (1mM) significantly inhibited SNP induced relaxation, while GLIB ($10^{-5}$M) was not able to affect the response to SNP. These results suggested that NO play an important role in the relaxation of ileal, and these effects are mediated via cGMP, voltage-dependent ($K_V$), inward rectifier ($K_{IR}$) and $Ca^{+2}$-dependent ($K_{Ca}$) channels.**

***Keywords:*** Nitric oxide, Potassium channels, cGMP, Ileal, Organ bath.

## INTRODUCTION

NO is the classical example of a simple molecule endowed (Wink *et al.*, 1998). Since the 1980, the free radical, NO, was discovered to be a crucial signaling molecule, with critically important physiological actions (Miller and Megson, 2007). It was found by Furchgott, Murad, and Iguarro who were honored for their discovery and being awarded the Nobel Prize for medicine in 1998 (Kasparek *et al.*, 2008; Kashiba *et al.*, 2002). It is a small hydrophobic molecule with chemical properties that make it uniquely suitable as both an intra- and intercellular messenger (Moncada and Higgs, 1993). Furthermore, it is free radicals and superoxide with short half-life (Estevez and Jordan, 2002), and ability to diffuse across biological membranes and through the cytoplasm (Flatley *et al.*, 2005).

Endogenous NO is generated in tissue from amino acid L-Arginine by nitric oxide synthases (NOS) (Huerta *et al*., 2008). Three isoforms of NOS are recognized depending on the tissue: neuronal (nNOS or Type 1) and endothelial (eNOS or Type 3) are constitutive calcium ($Ca^{+2}$) dependents and presents in the neural tissue and in the vascular endothelium, respectively, whereas inducible NOS (iNOS or Type 2) is $Ca^{+2}$-independent and is induced by bacterial endotoxins and cytokines in macrophages, smooth muscle, liver fibroblast and neutrophils (Kochar *et al*., 2011). However, inducible NOS (iNOS) are responsible for the enzymatic production of NO in the gastrointestinal tract (GIT) (Duncan, *et al.*, 1995). In addition to endogenous sources of NO, various exogenous NO donors are pharmacological active substances that, *in vivo* or *in vitro*, release NO (Yamamoto and Bing, 2000). However, the organic nitrates, nitrites, nitroso compounds and a variety of other nitrogen-oxide containing substances, including nitroprusside lead to the formation NO (Agasti, 2011), but the commonly used agents are the organic nitrates such as SNP, S-nitroso-N-acetylpenicillamine (SNAP), and glyceryl trinitrate (GTN; nitroglycerin) (Willmot and Bath, 2003). SNP is well known to function as an NO donor, since NO is releases indirectly from the nitroprusside group, probably via the S-nitrosation of thiolate groups by the nitroprusside, followed by the hemolytic decomposition of the resulting S-nitroso species to give NO gas (Poole, 2008).

In the GIT, there is increasing evidence indicates that NO is an important inhibitory neurotransmitter, and appears to act as the final common pathway to mediate enteric smooth muscle relaxation (Takahshi, 2003). The mechanism of NO action is not fully understood, but many of its actions are mediated by the activation of soluble guanylate cyclase (sGC), which results in an increase in the concentration of cyclic guanosine monophosphate (cGMP) in

smooth muscle (Ignarro *et al*., 1986), cGMP is thought to act directly on ion channels, and to activate protein kinase, which regulates the activity of proteins through phosphorylation (Robertson *et al*., 1993). Furthermore, the variability in the electrical signal in smooth muscles, generally based on the dynamic balance between outward $K^+$ current and inward $Ca^{+2}$ current, provides a range of gradation of activity for internal organs that permits normal function (Sperelakis, 2001). $K^+$ channels are the most widely distributed type of ion channel and are found in virtually all living organisms (Littleton and Ganetzky, 2000), since dysfunctions of $K^+$ channels would induce many diseases, various studies toward their functions in physiologic and pathologic process have been extensively launched (Shen *et al.,* 2003). Therefore, they are play important roles in vital cellular signaling processes in both excitable and non-excitable cells (Shieh *et al.,* 2000). Thus, $K^+$ channels found in smooth muscle of the gut reflects the capacity of this system to fine tune the electrical activity of the syncytium to control intestinal rhythm (Liang *et al*., 2012).

The aim of this study was to investigate the involvement of the most important cGMP and four subtypes $K^+$ channels ($K_{ATP}$, $K_{V,}$ $K_{Ca}$ and $K_{IR}$) in the relaxation induced by NO of rat ileal.

## MATERIAL AND METHOD

### Tissue Preparation

Adult male albino rats *(Rattus rattus norvegius*) weighing 250-350g were prepared from the animal house of the Department of Biology, Faculty of Science/University of Zakho. The animals were anaesthetized and then approximately 10 cm of terminal ileal was removed, washed in Tyrodes solution **(**concentrations in gm/L: KCl 0.2, Sodium Chloride (NaCl) 8, D (+) Glucose 1, Sodium Bicarbonate (NaHCO3) 1, Magnesium Chloride ($MgCl_2.6H_2O$) 0.1, Calcium Chloride Dihydrate ($CaCl_2.2H_2O$) 0.20 and Sodium Dihydrogen Phosphate ($NaH_2PO_4$) 0.05**),** freed from mesenteric attachment and then ileal was cut into smaller segments, each one of segments approximately 2 cm in length.

### Measurement of Ileal Tension

Ileal segments were transfer to organ bath, containing 25 ml Tyrodes solution at $37C^o$ and bubbled with 95% $O_2$ and 5% $CO_2$ to set pH at 7.40. One end of the ileal segment was fixed to a glass tissue hook at the bottom of the bath and the other end was attached to force transducer, and coupled to the trans bridge and Power Lab Data Acquisition System (ML 870, Power Lab, AD Instrument, Sydney, Australia) and computer running chart software (Version7) for measurement isometric tension. After mounting the ileal segments, 1g resting tension was applied to each tissue. Then segments were allowed to stabilize in Tyrodes solution for 60 minutes. During that time, the solution was changed every 15 minutes.

### Protocol of Experiment

The ileal segments were precontracted by either ACh ($10^{-5}$ M) and KCl (60mM) to obtain the maximum response. Then, to determine a dose–response curve on the contractility of ileal segments, different concentrations of NO donor (SNP; $3\times10^{-8}$ to $3\times10^{-4}$ M) was added cumulatively every 2-3 minutes.

The integrated mechanical activity induced by ACh was higher than that induced by KCl in relaxation induced by SNP, thus in the remaining experiment, ACh ($10^{-5}$ M) was used for precontraction of ileal segments.

To examine the possible role of cGMP and $K^+$ channels in the relaxation induced by SNP in rat ileal smooth muscle**,** segments were preincubated for 20 minutes with MB (sGC blocker), GLIB (blocker of $K_{ATP}$), 4-AP (blocker of K**v**), $BaCl_2$ (blocker of $K_{IR}$) and TEA (blocker of $K_{Ca}$).

### Statistical analysis

The statistical analysis was performed using two-way analysis of variance (ANOVA) supported by Bonferroni test when carrying out pair wise comparison between the same doses of different groups. P-value less than 0.05 ($P<0.05$) were considered as statistically significant. All the graph, calculation and statistical analysis were performed using GraphPad Prism software version 5.0 for windows (Graph Pad Software, San Diego California USA).

## RESULTS

### Effect of SNP on ACh and KCl -induced Contraction

As shown in the figure (1), SNP at doses between ($1X10^{-6}$ to $3X10^{-4}$ M) showed more potent relaxant effect in ACh ($10^{-5}$M) than KCl (60mM) induced ileal smooth muscle contractions with $IC_{50}$'s of ACh $1.548X10^{-6}$M

($IC_{50}$ of CI 95% between $1.185X10^{-6}$ to $2.024X10^{-6}$ M) and $6.746X10^{-7}$M ($IC_{50}$ of CI 95% between $2.048X10^{-7}$ to $2.222X10^{-6}$ M), as

well as the percentage of relaxation were 74.997 ± 1.206% and 39.006 ± 3.436% respectively, table (1).

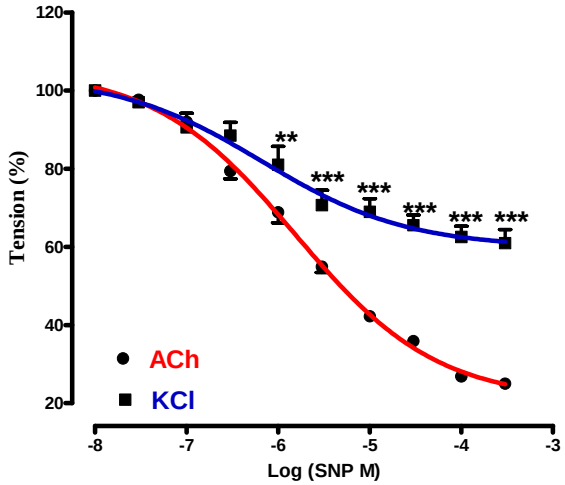


**Figure 1**: Cumulative dose-response curve for the effects of SNP on ACh ($10^{-5}$ M) and KCl (60mM) induced contractions in rats ileal

**Table1**: The $IC_{50}$ ($IC_{50}$ of CI 95%), and percentage of relaxation ± SEM for the effect of SNP in ACh- and KCl-induced contractions in rat ileal

| Treatments | ACh ($10^{-5}$M), n=7 | KCl (60mM), n=8 |
|---|---|---|
| **$IC_{50}$** | 1.548 X $10^{-6}$ | 6.746 X $10^{-7}$ |
| **$IC_{50}$ of CI 95%** | 1.185X$10^{-6}$ to 2.024X $10^{-6}$ | 2.048X$10^{-7}$ to 2.222X$10^{-6}$ |
| **Relaxation (%)** | 74.997 ±1.206 | 39.006 ± 3.436 |

## Effects of SNP with MB on the cGMP of smooth muscle cells from rat ileal

Preincubation of ileal segments for 20 minutes with MB (3mM) as a sGC blocker, showed SNP at doses between (3X$10^{-6}$ to 3X$10^{-4}$ M) highly significantly (P<0.001) reduced the relaxation ileal smooth muscle**,** with $IC_{50}$ of 4.671X$10^{-6}$M ($IC_{50}$ of CI 95% between 3.807X$10^{-7}$ to 5.731 X$10^{-5}$ M), and the percentage of relaxation reduced to 45.652 ± 6.373%, as shown in the figure (2) and table (2).

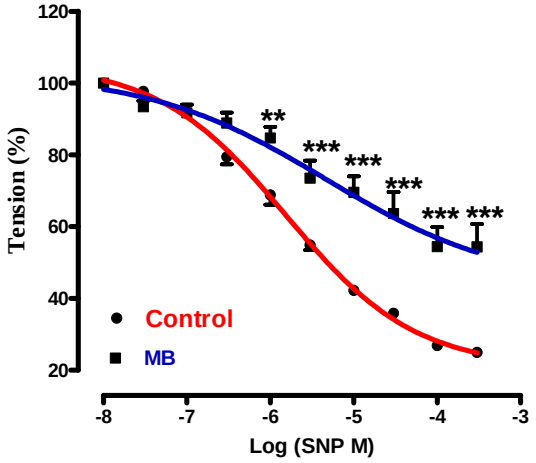


**Figure 2**: Cumulative dose-response curve for the relaxant effects of SNP on control and preincubated ileal segments with MB (3mM), pre-contracted with ACh ($10^{-5}$ M)

## Effect of $K^+$ Channels Blockers on the Inhibitory Response Induced by SNP

To investigate the possible role of the four types of $K^+$ channels in the development of relaxation induced by SNP, ileal segments preincubation for 20 minutes with individual GLIB ($10^{-5}$M), 4-AP (1mM), $BaCl_2$ (1mM) and TEA (1mM) as shown in figures (3, 4, 5 and 6) and table (2). GLIB was unable to modify the response of SNP, whereas 4-AP at a final dose (3 X $10^{-4}$ M; P<0.05) significantly reduced the relaxation induced by SNP. In contrast, $BaCl_2$ and TEA highly significantly (P<0.001) reduced the relaxation induced by SNP at doses between (1X$10^{-6}$ to 3X$10^{-4}$ M) and (3X$10^{-7}$ to 3X$10^{-4}$ M) respectively. The $IC_{50}$ of the treated segments with $K^+$ channel blockers GLIB, 4-AP, $BaCl_2$ and TEA were 1.542X$10^{-6}$ M ($IC_{50}$ of CI 95% between 6.199X$10^{-7}$ to 3.835X$10^{-6}$M), 5.945X$10^{-7}$ ($IC_{50}$ of CI 95% between 3.642X$10^{-7}$ to 9.705X$10^{-7}$ M), 1.866X$10^{-6}$ M ($IC_{50}$ of CI 95% between 8.131 X $10^{-7}$ to 4.280X$10^{-6}$ M), and 5.733X$10^{-5}$ M ($IC_{50}$ of CI 95% between 2.259X$10^{-7}$ to 0.0145 M) respectively, whereas the percentage of relaxation with four types of blockers were 66.179 ± 4.960%, 64.641 ± 5.602%, 50.437 ± 1.075%, and 49.443 ± 1.594%, respectively.

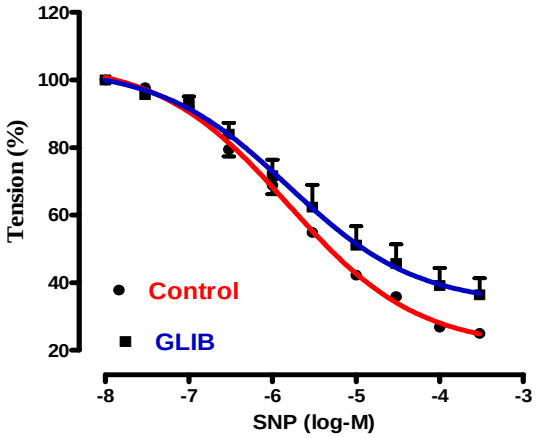


**Figure 3**: Cumulative dose-response curve for the relaxant effects of SNP on control and pre-incubated ileal segments with GLIB ($10^{-5}$ M) precontracted with ACh ($10^{-5}$ M)

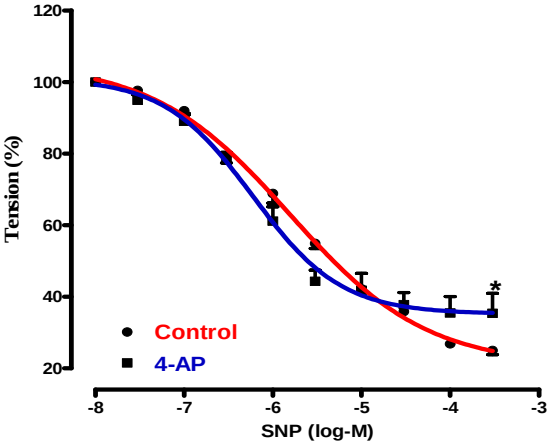


**Figure 4**: Cumulative dose-response curve for the relaxant effects of SNP on control and pre-incubated ileal segments with 4-AP (1mM), precontracted with ACh ($10^{-5}$ M)

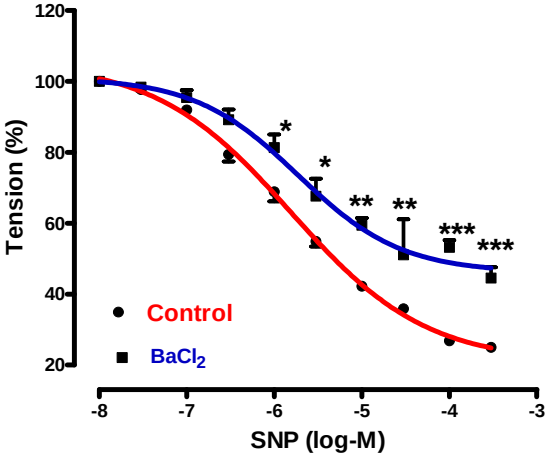


**Figure 5**: Cumulative dose-response curve for the relaxant effects of SNP on control and pre incubated ileal segments with $BaCl_2$ (1mM), precontracted with ACh ($10^{-5}$ M)

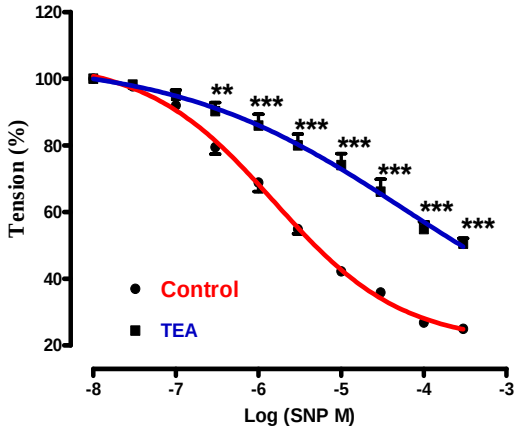


**Figure 6**: Cumulative dose-response curve for the relaxant effects of SNP on control and pre-incubated ileal segments with TEA (1mM), precontracted with ACh ($10^{-5}$ M).

**Table 2**: The $IC_{50}$ ($IC_{50}$ of CI 95%), percentage of relaxation ± SEM for the effect of SNP in control and pre-incubated ileal segments with methylene blue and $K^+$ channel blockers.

| Treatments | $IC_{50}$ | $IC_{50}$ of CI 95% | Relaxation (%) |
|---|---|---|---|
| **Control n= 7** | 1.548 X $10^{-6}$ | 1.185 X $10^{-6}$ to 2.024 X $10^{-6}$ | 74.997 ± 1.206 |
| **Methylene Blue (3mM) n=5** | 4.671 X $10^{-6}$ | 3.807 X $10^{-7}$ to 5.731 X$10^{-5}$ | 45.652 ± 6.373 |
| **GLIB($10^{-5}$ M) n= 8** | 1.542 X $10^{-6}$ | 6.199 X $10^{-7}$ to 3.835 X$10^{-6}$ | 66.179 ± 4.960 |
| **4-AP (1mM) n= 8** | 5.945 X $10^{-7}$ | 3.642 X $10^{-7}$ to 9.705X$10^{-7}$ | 64.641 ± 5.602 |
| **$BaCl_2$ (1mM) n= 5** | 1.866 X $10^{-6}$ | 8.131X $10^{-7}$ to 4.280X$10^{-6}$ | 50.437 ± 1.075 |
| **TEA (1mM) n= 6** | 5.733 X $10^{-5}$ | 2.259X$10^{-7}$ to 0.0145 | 49.443 ± 1.594 |

## DISCUSSION

It is well known that in GIT, ACh induces contraction through receptors and intracellular messengers, whereas KCl acts through ionic channels (Grasa, *et al.*, 2005). Moreover, ACh induced contractions in the rat ileal involve two different mechanisms coupled to muscarinic receptors. One mechanism activates non-selective cation channels in the plasma membrane, which results in membrane

depolarization. The depolarization stimulates $Ca^{+2}$influx through voltage-gated $Ca^{+2}$channels (VGCCs). The other mechanism activates contraction by the release of intracellular $Ca^{+2}$ (Kishore and Rahman, 2012). In contrast, a high-$K^+$ medium could depolarize the cellular membrane of ileal smooth muscle (Zhang, *et al*., 2005) by increase in $Ca^{+2}$ influx through voltage-dependent $Ca^{+2}$ channels (VDCCs) (Kaya, *et al.*, (2002); Borrelli, *et al.*, (2006) and Naseri, *et al.*, (2008). Furthermore, KCl has long been used as a convenient stimulus to bypass G protein-coupled receptors (GPCR) and activate smooth muscle by VDCCs that leads to increases in cytosolic free $Ca^{+2}$ ($[Ca^{+2}]i$), $Ca^{+2}$-calmodulin dependent myosin light chain (MLC) kinase activation, MLC phosphorylation and contraction (Ratz, *et al.*, 2005).

However, the present study showed that the contractile activity induced by ACh was higher than that induced by KCl with inhibitory effect of SNP in rat ileum. These results were agree with the results reported by Grasa *et al*., (2005), they found that the relaxant effect of SNP in longitudinal and circular muscle of rabbit small intestine by ACh higher than that induced by KCl.

In the current study, MB is the inhibitor of sGC, high significantly inhibited SNP induced relaxation. This suggests that the relaxant effects of NO may be act via activation of sGC in the rat ileal smooth muscle cells. This result is supported by previously reported studies of Zyromski *et al*., (2001) and Ueno *et al*., (2004) they showed that NO activates sGC in longitudinal muscle of human and mouse jejunum, as well as in circular muscle of rat jejunum (Vanneste *et al*., 2004). sGC activation was also reported to play a role *in vivo* intestinal motility (Patil, *et al*., 2005), as well as several hypotheses suggested for possible NO release from nerves in the gut wall, and then its diffuses through the cell membrane smooth muscle (Lecci, *et al*., 2002 and Takahshi, 2003), and binds to the heme moiety of the soluble enzyme sGC (Feelisch, 2008), resulting in the production of the cGMP from GTP which causes relaxation of smooth muscle cells (Ahern, *et al*., 2002); (Kanada, *et al*., 1992); (Ijoma, *et al*., 1995) and (Chung, *et al*., 2005).

Previous studies by Grasa *et al*., (2005) showed that the relaxation of smooth muscle induced by SNP of rabbit small intestine was not depended on the $K_{ATP}$ channels. Also in our study, when GLIB as a blocker of $K_{ATP}$ channels was added to the preparations, the SNP relaxations were not changed as compared with control segments. While preincubation of ileal segments with 4-AP as a blocker of Kv channels, caused weak inhibition on SNP-induced relaxation, because it caused inhibition due to NO only at a final dose. Koh *et al*., (1995); Lang and Watson (1998) they observed that Kv in colonic smooth muscle play an important role in NO-mediated relaxation.

It has been shown from the present study, that the $BaCl_2$ as a blocker of $K_{IR}$ channels, highly significantly reduced SNP-induced inhibitory effects. Also our results showed that the TEA as a blocker of $K_{Ca}$, highly significantly reduced SNP-induced relaxant effects in rat ileal. These results were supported with the results reported by Zizzo *et al*., (2005) and Grasa, *et al.* (2005) they found that the blocking of $K_{Ca}$ channels reduced inhibitory effects by SNP. Zizzo *et al.* studied mouse smooth muscle (longitudinal), while Grasa, *et al*. studied longitudinal and circular smooth muscle segments from rabbit ileal.

Although the mechanism action of NO in the relaxation of ileal smooth muscles remain poorly understood (Grasa, *et al.*, 2005), but in the light of the results obtained from the present work, observed that NO inhibited contractile activity of ileal smooth muscle through pathways do not mediated by the $K_{ATP}$ channels, but this action may be mediated by the activation of sGC resulting in the production of the cGMP which decreased intracellular $Ca^{+2}$, and leads to cellular hyperpolarization depended on the activation of Kv, $K_{IR}$ and $K_{Ca}$ channels. This study concluded that exogenous NO decreases the contractility of smooth muscle in ileal rats. This action is mediated by cGMP and three $K^+$ channels subtypes (Kv, $K_{IR}$ and $K_{Ca}$).

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## التأثيرالأرتخائي لنيترات الأوكسجين الواهب على نشاط التقلصي للفائفي: دور cGMP وقنواة البوتاسيوم

### الخلاصة

النواقل الغازية عبارة عن غازات ذاتية المنشأ وتلعب أدوارا مهمة في الوظائف الفسلجية. فكما هو معروف أن غاز نيترات الأوكسجين (NO) له تأثيرأرتخائي على القنواة المعوية. الهدف من الدراسة كان لتحديد مساهمة cGMP وقنواة البوتاسيوم (K+ channels)في الأرتخاء المستحدث من قبل غاز نيترات الأوكسجين على العضلات الملساء اللفائفي. حيث لوحظ أن التأثير الأرتخائي ل Sodium nitroprusside (SNP) وعند الجرعة ما بين (١×١٠$^{-٦}$ الى ٣ ×١٠$^{-٤}$ مول) على تقلص العضلة الملساء اللفائفي المستحدثة بالأستايل كولين (ACh) (١٠$^{-٥}$ مول) أكثر تأثيرا بالمقارنة مع تقلص العضلة بكلوريد البوتاسيوم (KCl) (٦٠ملي مول). كما أن قطع اللفائفي ولمدة ٢٠ دقيقة قبل تقلص العضلة بالأستايل كولين عوملت بأنواع مختلفة من المثبطات مثل Methylene blue (MB) ، Glibenclamide (GLIB)، 4-AP) 4-aminopyridine، Barium Chloride (BaCl2) و Tetraethylammonium (TEA).
حيث لوحظ أن لمثبط MB (٣ ملي مول) , 4-AP (١ملي مول) , $BaCl_2$ (١ملي مول) وTEA (١ملي مول) تأثيرا معنويا على أرتخاء العضلة المستحدثة من قبل (SNP) , بينما لم تتمكن مثبط GLIB (١٠$^{-٥}$ مول) من التأثيرعلى أستجابة (SNP).فمن المعتقد وحسب تلك النتائج, أن لنيترات الأوكسجين دورا مهما في أرتخاء اللفائفي وذلك أعتمادا على cGMPوقنواة البوتاسيوم من نوع Kv , $K_{IR}$ و $K_{Ca}$.

# کاریگەریا خابوونەوەی ئوکسیدێ نیترات پخشینە ر لسەر چالاکێی گرژبوونا پیچاوپیچ: رولێ cGMP وچنالێن پوتاسیوم


**پوختە**

ڤەگوهێزەرێن گازی بریتینە ژ گازێن پەیدابوونا وان خویی، ورولێن گرنگ دگێرن د فرمانێن فسیولوجیدا. هەروەکی دیار گازا ئوکسیدێ نیترات (NO) کارتیکرنەکا خاڤبوونی لسەر جوکێن ریڤیکان هەیە. ئەڤ ڤەکولینە هاتە ئەنجامدان بۆ تاقیکرنا پشکداری کرنا cGMP وچنالێن پوتاسیۆمی دوێ خاڤبوونێدا ئەوا ژ ئەگەرێ گازا نیترات ئوکسجینێ پەیدابووی لسەر ماسولکێن لووسی د پێچاوپێچا دا. هاتە دیتن کو کاریگەریا خاڤبوونێ یا Sodium nitroprusside (SNP)دخەستیێن دناڤبەرا (١×١٠$^{-٦}$ تا ٣ ×١٠$^{-٤}$ مول) لە سەر گرژبوونا ماسولکین پیچاوپیچ ئەوا ب رێکا ئەستایل کولین (١٠$^{-٥}$ مول) هاتیە ئەنجامدان یا ب هێزتر بوو ب بەراوردی دگەل کلوریدێ پوتاسیومی (٦٠ ملی مول). هەروەسا پارچین پێچاوپێچ وبو ماوەی (٢٠) خولەکان بەری گرژبوونا ماسولکێ ب ئەستایل کولین بە چەند جۆرین جیاواز ژ راوەستینەران هاتە تێکرن وە ك (MB) Methylene blue، (GLIB) Glibenclamide، (4-AP) 4-aminopyridine، Barium Chloride (BaCl2) و Tetraethylammonium (TEA). هاتە دیتن راوەستینەری MB (٣ ملی مول), 4-AP (١ملی مول) , $BaCl_2$ (١ملی مول) و TEA (١ملی مول) کاریگەریێ لوجستی تومارکرن د وێ خاڤبوونا ماسولکەدا ئەوا ب رێکا (SNP) هاتیە کرن، لێ دبەرامبەردا هاتە دیتن راوەستێنەری GLIB (١٠$^{-٥}$ مول) چ کاریگەریێ لە سەر کارتیکرنا (SNP) نە بوو. لە دویف وان ئەنجامان هاتە دیارکرن, گازا ئوکسیدێ نیترات رولێکی گرنك هەبوو د خاڤبوونا پێچاوپێچا دا بە پشت لە سەر cGMP وچنالێن پوتاسیۆمی ژ جۆری Kv، $K_{IR}$ و $K_{Ca}$.