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# Vasoconstriction triggered by hydrogen sulfide: Evidence for $Na^+$,$K^+$, 2$Cl^-$cotransport and L-type $Ca^{2+}$ channel-mediated pathway

Sergei N. Orlov[a,c,*], Svetlana V. Gusakova[b,**], Liudmila V. Smaglii[b], Svetlana V. Koltsova[c], Svetalana V. Sidorenko[c]

[a] Central Research Laboratory, Siberian State Medical University, Moskovskiy trakt 2, Tomsk 634050, Russia
[b] Department of Biophysics and Functional Diagnostics, Medical and Biological Faculty, Siberian State Medical University, Moskovskiy trakt 2, Tomsk 634050, Russia
[c] Faculty of Biology MV Lomonosov Moscow State University, Leninskiye gory 1/12, Moscow 119991, Russia

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ABSTRACT

*Objectives:* This study examined the dose-dependent actions of hydrogen sulfide donor sodium hydrosulphide (NaHS) on isometric contractions and ion transport in rat aorta smooth muscle cells (SMC).

*Methods:* Isometric contraction was measured in ring aortas segments from male Wistar rats. Activity of $Na^+$/$K^+$-pump and $Na^+$,$K^+$,2$Cl^-$cotransport was measured in cultured endothelial and smooth muscle cells from the rat aorta as ouabain-sensitive and ouabain-resistant, bumetanide-sensitive components of the $^{86}Rb$ influx, respectively.

*Results:* NaHS exhibited the bimodal action on contractions triggered by modest depolarization ($[K^+]_o = 30$ mM). At $10^{-4}$ M, NaHS augmented contractions of intact and endothelium-denuded strips by ~ 15% and 25%, respectively, whereas at concentration of $10^{-3}$ M it decreased contractile responses by more than two-fold. Contractions evoked by $10^{-4}$ M NaHS were completely abolished by bumetanide, a potent inhibitor of $Na^+$,$K^+$,2$Cl^-$cotransport, whereas the inhibition seen at $10^{-3}$ M NaHS was suppressed in the presence of $K^+$ channel blocker TEA. In cultured SMC, $5 \times 10^{-5}$ M NaHS increased $Na^+$,$K^+$,2$Cl^-$ - cotransport without any effect on the activity of this carrier in endothelial cells. In depolarized SMC, $^{45}Ca$ influx was enhanced in the presence of $10^{-4}$ M NaHS and suppressed under elevation of [NaHS] up to $10^{-3}$ M. $^{45}Ca$ influx triggered by $10^{-4}$ M NaHS was abolished by bumetanide and L-type $Ca^{2+}$ channel blocker nicardipine.

*Conclusions:* Our results strongly suggest that contractions of rat aortic rings triggered by low doses of NaHS are mediated by activation of $Na^+$,$K^+$,2$Cl^-$cotransport and $Ca^{2+}$ influx via L-type channels.

## 1. Introduction

Nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide ($H_2S$) are gasotransmitters synthesized endogenously from arginine, glycine-derived heme and cysteine, respectively. They freely penetrate across the plasma membrane and trigger cell signaling in a receptor-independent manner. In the cardiovascular system, $H_2S$ is mainly produced in vascular smooth muscle cells (SMC), periventitial adipose tissue, and erythrocytes by cystathionine-β-synthetase, cystathionine-γ-lyase (CSE) and 3-mercaptosulfurtransferase [1,2].

Numerous research teams reported that the application of $H_2S$ donor sodium hydrosulphide (NaHS) leads to dilatation in blood vessels of different origin [1,3,4]. In contrast to NO and CO the vasorelaxant effect of NaHS is not mediated by the cGMP pathway [5,6]. $H_2S$ activates ATP-sensitive $K^+$ channels ($K_{ATP}$) and/or $Ca^{2+}$-activated $K^+$ channels ($K_{Ca}$) [1,7]. Indeed, $H_2S$-induced vasodilatation was mimicked by $K_{ATP}$ openers pinacidil diazoxide, and abolished by glibenclamaide and other inhibitors of $K^+$ channels [3,4]. Vasorelaxative actions of $H_2S$ might also be mediated by partial inhibition of phosphodiesterase activity [8].

Al-Magableh and Hart demonstrated that NaHS-induced relaxation of mouse aorta was not affected by the removal of endothelium [9] thus

*Abbreviations:* NaHS, sodium hydrosulphide; NO, nitric oxide; CO, carbon monoxide; $H_2S$, hydrogen sulfide; CSE, cystathionine-γ-lyase; NaHS, sodium hydrosulphide; sGC, soluble guanylyl cyclase; cGMP, cyclic guanosine monophosphate; $K_{ATP}$, ATP-sensitive potassium channels; $K_{Ca}$, $Ca^{2+}$-activated potassium channels; EDHF, endothelium-derived hyperpolarizing factor; SMC, smooth muscle cells; VSMC, vascular smooth muscle cells; PE, phenylephrine; NKCC, $Na^+$,$K^+$,2$Cl^-$ cotransport; PSS, physiologically-balanced salt solution; VSMR, vascular smooth muscles from rat; EC, endothelial cells; TEA, tetraethylammonium chloride; RAEC, endothelial cells from rat aorta; RASMC, smooth muscle cells from rat aorta; COX, cyclooxygenase

* Corresponding author at: Faculty of Biology MV Lomonosov Moscow State University, Leninskiye gory 1/12, Moscow 119991, Russia.
** Corresponding author.
*E-mail addresses:* sergeinorlov@ya.ru (S.N. Orlov), gusacova@yandex.ru (S.V. Gusakova).

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indicating vascular SMC as a major target of $H_2S$. This conclusion, however, contradicts the data obtained in mice with a target deletion of the gene encoding CSE. Yang and co-workers found that *CSE*$^{-/-}$-mice display pronounced hypertension and diminished endothelium-dependent vasorelaxation [10]. More recently, it was shown that CSE knockout mice exhibited elevated resting membrane potential in the SMC of mesenteric arteries but not that of the aorta thus fulfiling the role of $H_2S$ in peripheral resistant blood vessels as an endothelium-derived hyperpolarizing factor (EDHF) [11].

$H_2S$ actions are complex, showing great species and vascular bed differences. Several research groups reported that NaHS at low concentrations exhibited contractile activity in norepinephrine-treated rat pulmonary arteries [12], in rat and mouse aortic rings precontracted with phenylephrine (PE) and elevated $[K^+]_o$ [13], in PE-treated rat gastric arteries [14] and human internal mammary arteries [15]. In contrast to relaxant actions, elevation of PE-induced contraction by low doses of NaHS was abolished in endothelium-denuded mouse aorta [13] as well as in mouse aorta and rat gastric artery treated with inhibitors of NO synthase, soluble guanylate cyclase (sGC) and cyclooxygenase (COX) [13,14]. NaHS-induced contraction was also stronger in the acetylcholine-prerelaxed human internal mammary artery suggesting inhibition of NO- and EDHF-induced relaxation [15].

Previously, we found that ubiquitous isoform of $Na^+$,$K^+$,$2Cl^-$ -cotransporter (NKCC1) affects vascular SMC (VSMC) contraction via regulation of intracellular $Cl^-$ concentration, electrical membrane potential and $Ca^{2+}$ influx mediated by voltage-gated L-type $Ca^{2+}$ channels [16,17]. In preliminary experiments we have also shown that NaHS triggers constriction in endothelium-denuded vascular segments that was diminished by NKCC inhibitor [18]. In this study we expanded examination of the role of ion transporters in $H_2S$-induced signaling by comparing the dose-dependent actions of NaHS on the contractions of intact and endothelium-denuded rat aortic rings and inwardly-directed $K^+$ and $Ca^{2+}$ transport in cultured SMC and endothelial cells (EC).

## 2. Material and methods

### 2.1. Preparation of aortic rings

Endothelium-denuded aortic rings were obtained from the thoracic aorta of 11- to 13-week-old Wistar rats euthanized under deep intraperitoneal anaesthesia with sodium pentobarbital (Nembutal, 70 mg/kg) in accordance with institutional animal care guidelines. The isolated aorta was placed in physiologically-balanced salt solution (PSS) containing 120.4 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.5 mM glucose and 15 mM tris-HCl (pH 7.4). Connective tissue and fat were taken out with scissors whereas the endothelium was removed by careful rotation of a wooden manipulator inside the VSMR lumen just before the experiments. The 2- to 3-mm aortic rings were either used immediately or stored at 4 єC for up to 24 h. In preliminary experiments, we documented that 24-h storage did not affect aortic ring contractile responses.

### 2.2. Measurement of aortic ring tension

Isometric contraction of aortic rings was measured using four-channel Tissue Bath System Myobath II. Aortic rings were mounted in 10-ml baths with stainless steel hooks inserted into the vascular ring orifice. One hook was fastened to a mechanical force transducer. The tissues were bathed in PSS at 37 °C and bubbled with room air at a volumetric speed of ~1 ml/min. To control the contractile response, the rat vascular smooth muscles (VSMR) were equilibrated for 1 h at tension of 0.5–1 g and exposed to $K_o^+$-induced depolarization caused by isosmotic substitution of NaCl with KCl. The responses to NaHS were expressed in percentage of the contraction obtained with 30 mM KCl, which was taken as 100%. In part of experiments aortic rings were treated with 10 μM bumetanide or10 mM tetraethylammonium chloride (TEA).

### 2.3. Cultured cells

The precise measurement of cell volume and inward ion fluxes in VSMR are complicated by a relatively large extracellular space, the presence of fibroblasts, and VSMC heterogeneity. On the other hand, long-term cultured VSMC rapidly down regulate the expression of several specific genes that define their contractile phenotype in vivo. Keeping this in mind, we employed VSMC isolated from rat aorta and maintained in culture in accordance with previously published protocol up to passages 3–8 [19]. Endothelial cells from rat aorta (EC) were kindly provided by Dr. Thorin-Trescases (Institute of Cardiology, University of Montreal, Canada). These cells were isolated and passaged 3–4 times as described elsewhere [20,21]. To establish quiescence, VSMC were incubated before experiments during 24 h in the presence of 0.2% calf serum.

### 2.4. Measurement of $^{86}Rb$ and $^{45}Ca$ influx

Activity of $Na^+/K^+$-pump and $Na^+$,$K^+$,$2Cl^-$ – cotransporter was measured as an ouabain-sensitive and ouabain-resistant, bumetanide-sensitive components of the $^{86}Rb$ influx, respectively. The cells seeded in 24-well plates were washed twice with 2-ml aliquots of *medium A* containing 150 mM NaCl and 10 mM HEPES-tris buffer (pH 7.4). Then, the medium was aspirated and 0.25 ml of *medium B* containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES-tris (pH 7.4) and tested compounds were added. It was followed by 10 min incubation at 37 °C, 0.25 ml of medium B containing 1–2 μC/ml $^{86}Rb$ ± ouabain and bumetanide at concentrations of 3 mM and 20 μM, respectively. To measure $^{45}Ca$ influx, washed cells were incubated during 30 min in 1 ml medium B and 10 min in medium B containing 0.2 mM $CaCl_2$ ± 2 μM nicardipine and other compounds indicated in the Table 1 legends. Thereafter, each well was supplemented with 0.25 ml of prewarmed medium containing 4 μCi/ml $^{45}Ca$ and NaCl and KCl at total concentration of 150 mM. In preliminary experiments, we found that $^{86}Rb$ and $^{45}Ca$ uptake by VSMC from rat aorta and EC from rat aorta is linear up to 25 and 10 min, respectively. Considering this, $^{86}Rb$ and $^{45}Ca$ uptake was terminated in 10 and 5 min, respectively, by the addition of 2 ml of ice-cold *medium W* containing 100 mM $MgCl_2$ and 10 mM HEPES-tris buffer (pH 7.4). The cells were washed 3 times with ice-cold medium W and radioactivity of the incubation medium and cell lysate was measured with a liquid scintillation analyzer. The rate of ion influx ($V$) was calculated as $V = A/am$, where $A$ was the radioactivity of the samples (cpm), $a$ was the specific

**Table 1**
Effect of NaHS, bumetanide, TEA and nicardipine on the rate of 45Ca influx in cultured smooth muscle cells.

| Additions, μM | $^{45}Ca$ influx, pmol/mg protein/5 min | |
|---|---|---|
| | $[K^+]_o = 5$ mM | $[K^+]_o = 30$ mM |
| 1. None (control) | 100 | 278 ± 23* |
| 2. Nicardipine, 2 | 121 ± 15 | 96 ± 11# |
| 3. Bumetanide, 10 | 106 ± 13 | 186 ± 28*,# |
| 4. TEA, $10^4$ | 87 ± 9 | 268 ± 18* |
| 5. NaHS, 100 | 110 ± 20 | 387 ± 25*,# |
| 9. NaHS, 1000 | 106 ± 15 | 177 ± 18*,# |

Means ± S.E. obtained in 5 independent experiments performed in quadruplicates are shown. The rate of $Ca^{2+}$ influx at $[K^+]_o$ = 5 mM and in the absence of any additions listed in the left column varied from 389 and 425 nmol / mg protein / 5 min was taken as 100%.

* $p < 0.05$ compared to $^{45}Ca$ influx at $[K^+]_o$ = 5 mM.

\# - $p < 0.05$ compared to control at $[K^+]_o$ = 30 mM (at $[K^+]_o$ = 5 mM, there was no significant differences of $Ca^{2+}$ influx in the presence of additions in comparison with control).

radioactivity of $K^+$ ($^{86}Rb$) or $^{45}Ca$ (cpm/nmol), and *m* was protein content measured with the modified Lowry method. For more details, see [22,23].

### 2.5. Chemicals and statistics

$^{86}RbCl$ and $^{45}CaCl$ was from Perkin Elmer (Waltman, MA, USA). The rest of chemicals were obtained from Sigma (St. Louis, MO, USA) and Serva (Heidelberg, Germany). The stock solution of bumetanide (20 mM) was prepared in DMSO. The stock solution of NaHS was prepared in distalled water immediately before use. The maximal concentrations of $H_2S$ in the solutions of NaHS may be calculated according to the formula: $[H_2S] = [NaHS]/(1 + 10^{-pK}/10^{-pH})$ [24–26], where the pK at 37 °C is 6.755 [26] and the pH of the solution is 7.4. Earlier studies [27–30] demonstrated that at pH of 7.4 and 37 °C about 20% of sulfide is present as $H_2S$ $H_2S$. According to previous data, solutions prepared by bubbling $H_2S$ gas and by dissolving NaHS produce the similar effects on smooth muscle contraction [31–33].

The data, presented as means ± SE, were analyzed by Student's *t*-test or the *t*-test for dependent samples, as appropriate. Significance was defined as $p < 0.05$.

## 3. Results

### 3.1. Effect of NaHS on aortic ring contractions

We did not detect any significant impact of NaHS in the range from $5\times10^{-6}$ till $10^{-3}$ M on the baseline tension of endothelium-denuded aortic rings (data not shown). Fig. 1 displays that under partial depolarization triggered by elevation of KCl up to 30 mM, NaHS exhibit the bimodal action: in the range from $5\times10^{-6}$ to $10^{-4}$ M this compound dose-dependently increases contractions by ~ 25% (127.5 ± 5.7% (n = 6; $p < 0.05$) of KCl-induced contraction) whereas further elevation of its concentration up to $10^{-3}$ M decreased the maximal tension by ~ 2-fold (48.3 ± 5.0% (n = 6; $p < 0.05$) of KCl-induced contraction). Stronger depolarization in the presence of 60 mM, 90 mM and 120 mM KCl attenuated both contractile and inhibitory responses of NaHS (Fig. 1**A**). The inhibitory action of NaHS was also revealed in endothelium-denuded aortic strips precontracted by $\alpha_1$-adrenomimetic phenylephrine (Fig. 1**B**). Previously we reported that high-ceiling diuretic bumetanide suppresses contractions of rat aorta and mice mesenteric arteries triggered by phenylephrine and modest depolarization in the presence of 30 mM KCl but does not affect contractions triggered by higher depolarization in the presence of 60 mM KCl. Ubiquitous $Na^+,K^+,2Cl^-$ - cotransporter NKCC1 is the only isoform of this carrier expressed in smooth muscle and endothelial cells [34]. Data obtained in genetically engineering mice demonstrated that bumetanide affect smooth muscle contraction by inhibition of NKCC1, attenuation of $[Cl^-]_i$, smooth muscle hyperpolarization and inhibition of $Ca^{2+}$-influx via L-type $Ca^{2+}$ channels [16,17,35]. Keeping this observation in mind, in the rest of experiments we investigated the role of $Na^+,K^+,2Cl^-$ cotransporter in the bimodal action of NaHS on aortic strips precontracted by 30 mM KCl.

Acetylcholine suppressed baseline contraction of intact aorta (Fig. 2**A**) without any significant impact on this parameter in endothelium-denuded aortic rings (Fig. 2**C**). Unlike in endothelium-denuded aorta (Fig. 2**D**), NaHS at concentration of $10^{-5}$, $5\times10^{-5}$ and $10^{-4}$ M did not significantly affect contraction of intact aortic rings subjected to depolarization in the presence of 30 mM KCl whereas inhibitory actions of $5\times10^{-4}$ and $10^{-3}$ M NaHS were preserved (39.7 ± 4.3 % and 15.5 ± 7.5%, respectively, of KCl-induced contraction (n = 6; $p < 0.05$)) (Fig. 2**B**).

Considering the involvement of the NKCC1 in the contractile responses of SMC subjected to partial depolarization [16], we examined the action of a potent inhibitor of NKCC1 bumetanide on the bimodal modulation of aortic ring contraction by NaHS. Both in intact and endothelium-denuded aorta 10 μM bumetanide completely blocked contractile responses triggered by low doses of NaHS, whereas inhibitory actions of high doses of NaHS were preserved (Figs. 2**B** and 2**D**). We also observed that in endothelium denuded aorta bumetanide reversed contractile responses of $10^{-4}$ M NaHS (33.8 ± 5.8% (n = 6; $p < 0.05$)) and augmented inhibitory actions on contractile responses of higher doses of NaHS (19.0 ± 4.0% (n = 5, $p < 0.05$) of KCl-induced contraction) (Fig. 2**D**).

Compelling evidence indicates that $H_2S$-induced dilatation of distinct vascular beds is mediated by activation of $K_{ATP}$- and/or $K_{Ca}$-channels (for review, see [1,7]). Considering this, we employed TEA as non selective inhibitor of all $K^+$ channels studied so far. Fig. 3 shows

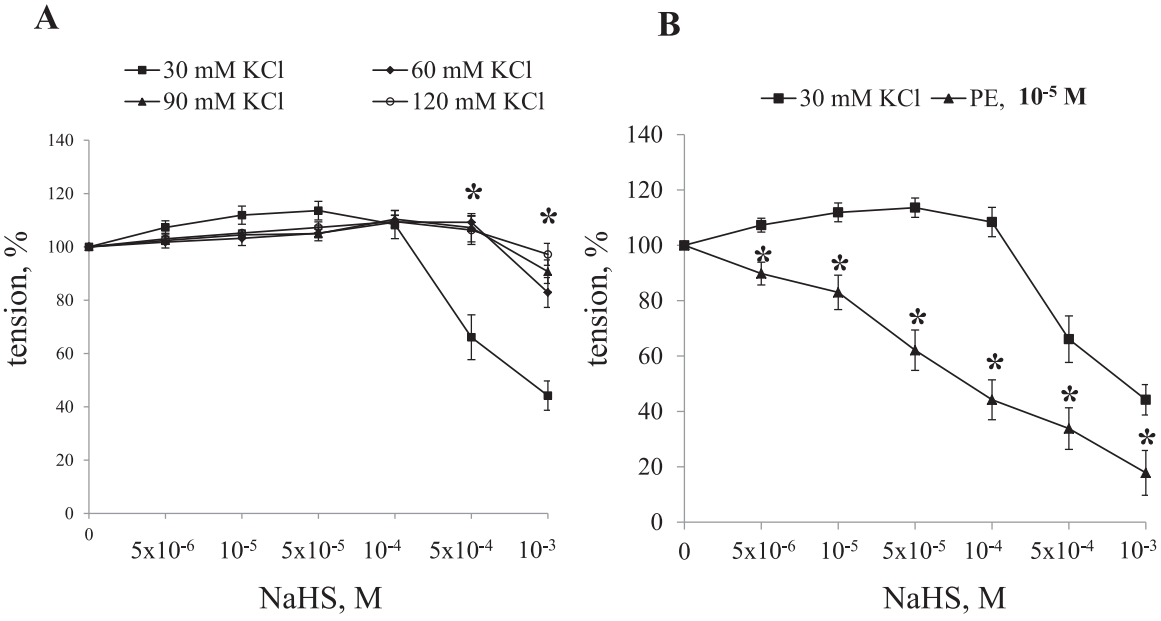


**Fig. 1.** Dose-dependent actions of NaHS on contractions of endothelium-denuded aortic rings at extracellular KCl concentration of 30, 60, 90 and 120 mM. and in the presence of 10 μM phenylephrine. Maximal contractions in the absence of NaHS were taken as 100%. Means and standard errors obtained in **6** independent experiments are shown. * - $p < 0.05$.

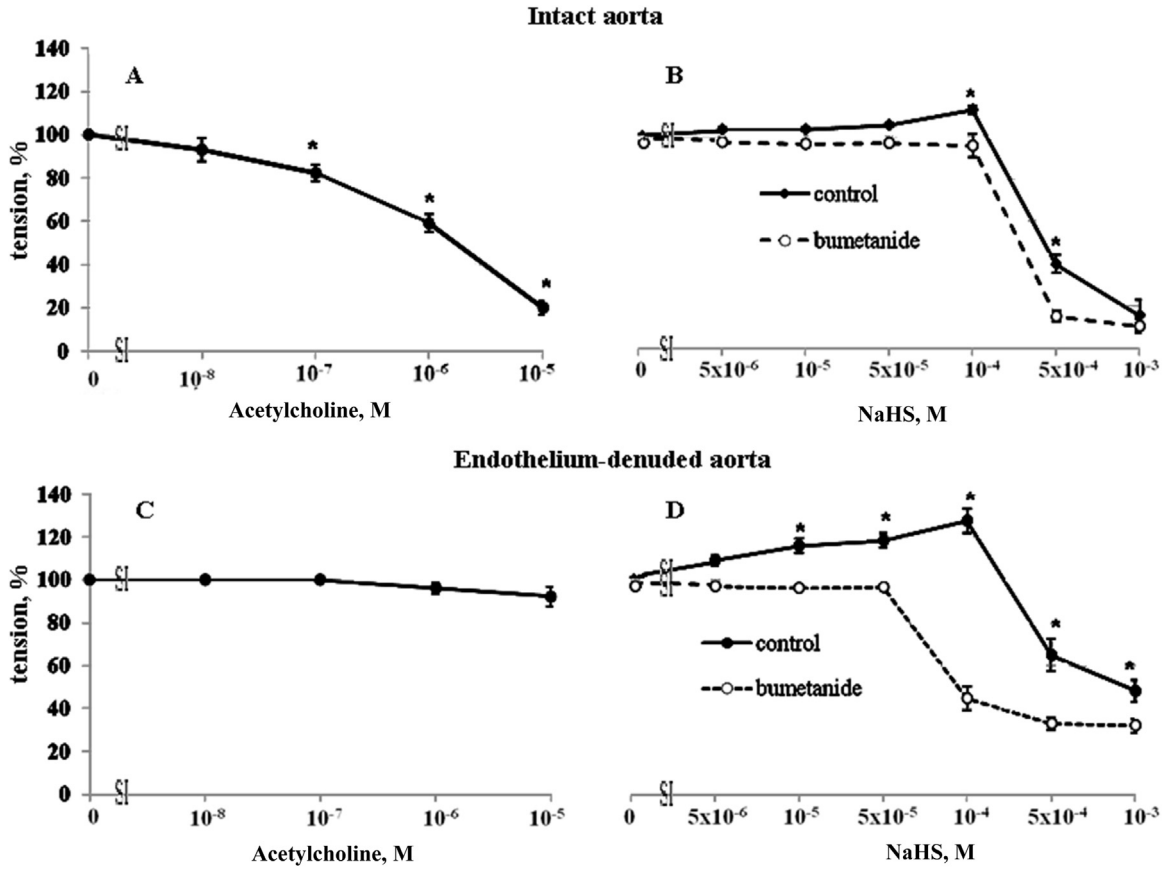


**Fig. 2.** Dose-dependent actions of acetylcholine (**A** and **C**) and NaHS (**B** and **D**) on contractions of intact (**A** and **B**) and endothelium-denuded (**C** and **D**) rat aortic rings in the presence and absence of $10^{-5}$ M bumetanide. Maximal contractions in the absence of acetylcholine and NaHS were taken as 100%. Means and standard errors obtained in **6** independent experiments are shown. * $p < 0.05$ compared to bumetanide-treated rings.

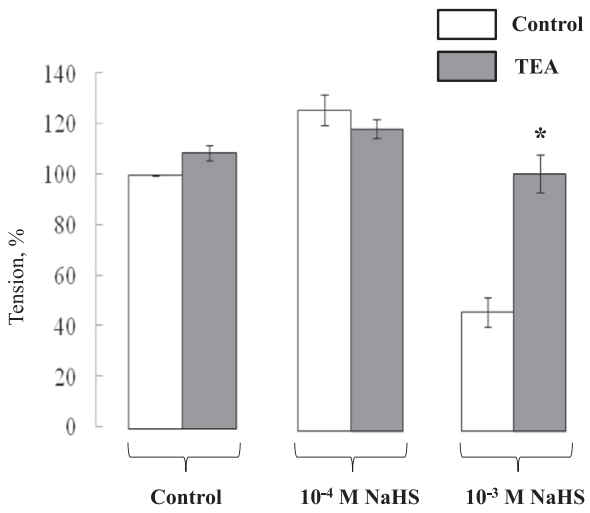


**Fig. 3.** Effect of TEA on contractions of rat aortic rings in the presence and absence of $10^{-4}$ M and $10^{-3}$ M NaHS. Contractions were evoked by elevation of $[K^+]_o$ up to 30 mM. Maximal contractions in the absence of NaHS were taken as 100%. Means and standard errors obtained in **6** independent experiments are shown. * - $p < 0.05$ compared to TEA-untreated rings.

that the inhibitory action of $10^{-3}$ M NaHS on aortic rings contractions was completely abolished (95.4 ± 7.1% (n = 6; $p < 0.05$ compared to TEA-untreated rings) in the presence of 10 mM TEA whereas contractions evoked by $10^{-4}$ M NaHS were preserved (116.7 ± 3.7% (n = 6; $p > 0.05$ compared to TEA untreated rings)).

### 3.2. Effect of NaHS on $K^+$ influx

Fig. 4**A** shows that in SMC, $Na^+$,$K^+$-pump and NKCC measured as a rate of ouabain- and bumetanide-sensitive $^{86}Rb$ influx, respectively, contributed to 55% and 34% of net $K^+$ uptake. These numbers are consistent with previously reported data [22,36]. Elevation of NaHS up to $5\times10^{-5}$ – $10^{-4}$ M dose-dependently increased NKCC (Fig. 4**B**). We did not observe any significant actions of NaHS on the activity of $Na^+/K^+$-pump as well on the passive permeability of SMC for $K^+$ estimated as (ouabain + bumetanide)-resistant component of the rate of $^{86}Rb$ influx.

Fig. 5A shows that in endothelial cells, $Na^+$,$K^+$-pump contributes to ~65% of the net $K^+$ ($^{86}Rb$) uptake. Elevation of NaHS from $5\times10^{-5}$ M to $5\times10^{-4}$ M resulted in ~80%- inhibition of the $Na^+$,$K^+$-pump (Fig. 5**B**) (Fig. 5**A**). Unlike SMC, we did not observe any activation of NKCC by low doses of NaHS in cultured endothelial cells from the rat aorta.

### 3.3. Effect of NaHS on $Ca^{2+}$ influx

Consistently with previous results [22,23], depolarization of SMC in high-$K^+$ medium resulted in elevation of $^{45}Ca$ influx by 2–3-fold that was completely abolished by inhibition of voltage-gated L-type $Ca^{2+}$ channel with nicardipine (Table 1). Addition of 10 μM bumetanide decreased depolarization-induced $^{45}Ca$ uptake by ~60% without significant impact on the baseline $^{45}Ca$ uptake. Unlike SMC, neither elevation of $[K^+]_o$ nor nicardipine and bumetanide affected the rate of $^{45}Ca$ influx in endothelial cells from the rat aorta (data not shown).

In control medium containing 5 mM $K^+$, neither $10^{-4}$ nor $10^{-3}$ M NaHS changed the rate of $^{45}Ca$ influx in SMC (Table 1). In depolarized

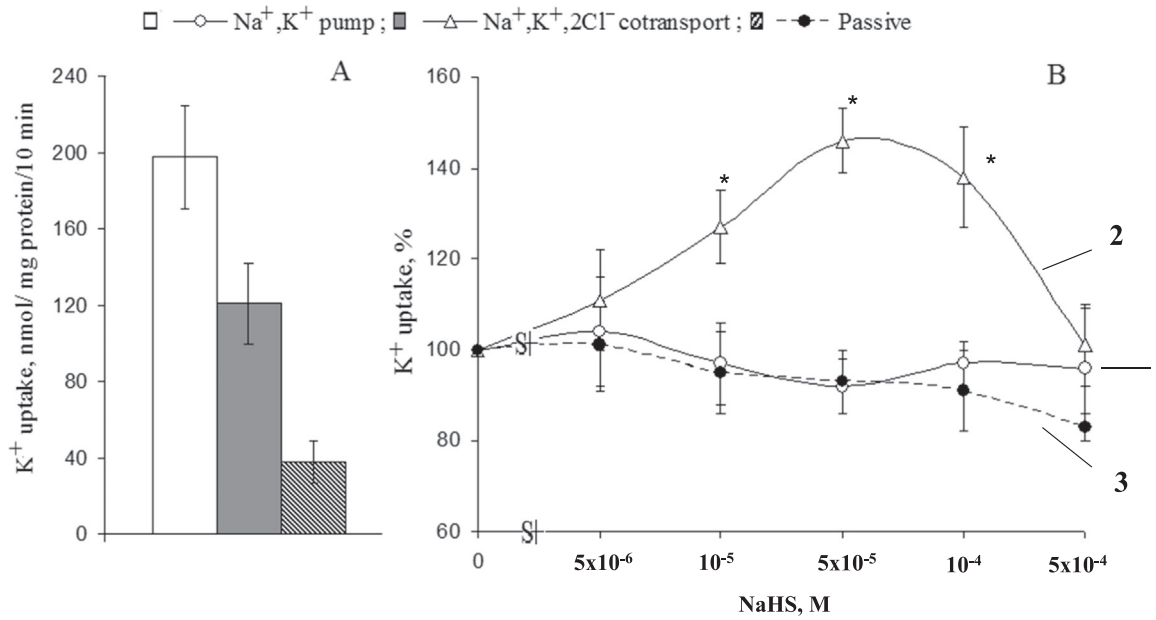


**Fig. 4.** $K^+$ ($^{86}Rb$) influx in smooth muscle cells from the rat aorta. **A**. Absolute values of the activity of $Na^+,K^+$-pump (ouabain-sensitive component of the rate of $^{86}Rb$-influx), NKCC (ouabain-resistant, bumetanide-sensitive component of the rate of $^{86}Rb$-influx) and passive permeability for $K^+$ (ouabain + bumetanide)-resistant $^{86}Rb$ influx). **B**. Dose-dependent actions of NaHS on $Na^+/K^+$-pump (**1**), NKCC (**2**) and the rate (ouabain + bumetanide)-resistant $K^+$ ($^{86}Rb$) influx (**3**). The values obtained in the absence of NaHS were taken as 100%. Means and standard errors obtained in **5** independent experiments are shown. * $p < 0.05$ compared to controls.

SMC, nicardipine-sensitive $^{45}Ca$ influx was further potentiated by $10^{-4}$ M NaHS whereas at a concentration of $10^{-3}$ M the $H_2S$ donor decreased this component of $^{45}Ca$ influx by ~2-fold. These results are in accordance with increment of nifedipine-sensitive $Ca^{2+}$ influx detected in Fura-3-loaded rat coronary artery treated with modest doses of NaHS [37]. We also observed that side-by side with nicardipine, the increment of depolarization-induced $^{45}Ca$ influx seen in the presence of $10^{-4}$ M NaHS was completely abolished by bumetanide but was insensitive to TEA (Table 2).

In contrast, the inhibitory action of high doses of NaHS on depolarization-induced $^{45}Ca$ influx was insensitive to bumetanide but was suppressed by TEA (Table 3).

## 4. Discussion

Our results show the excitatory and inhibitory actions of modest ($< 10^{-4}$ M) and high ($10^{-3}$ M) doses of NaHS on the contractions of rat aortic rings triggered by $K_o^+$-derived depolarization. These results are consistent with the bimodal action of NaHS on the baseline tension of rat aorta and portal vein [38], human internal mammary arteries [13,15], rat and mouse aortic rings precontracted with PE and elevated $[K^+]_o$ [13], PE-treated rat gastric [14] and rat pulmonary artery

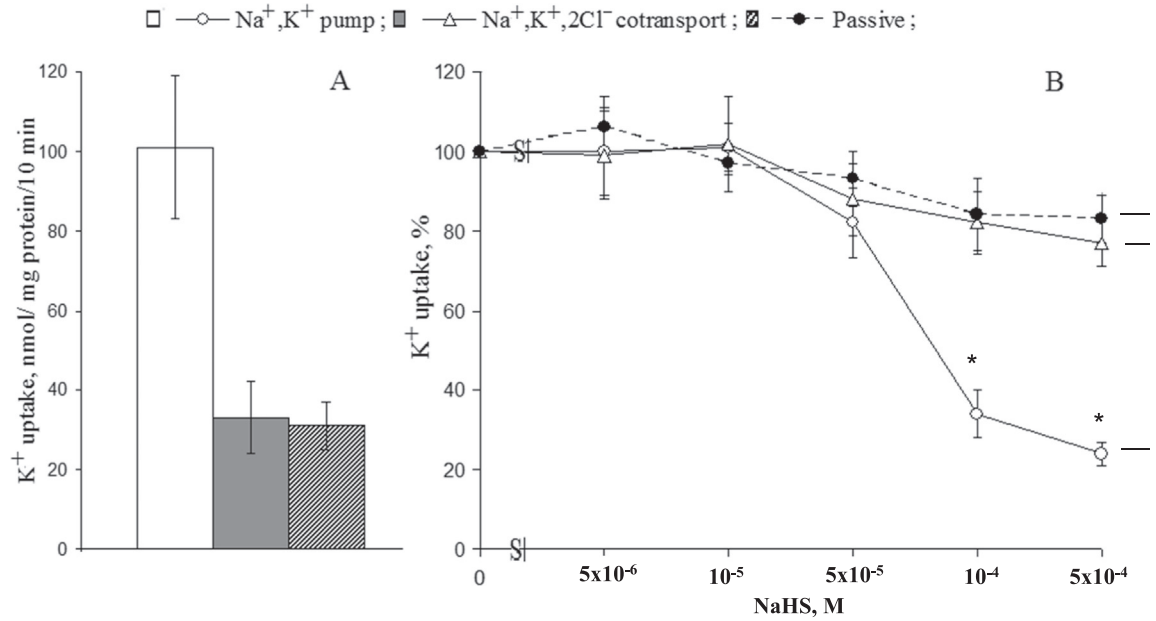


**Fig. 5.** $K^+$ ($^{86}Rb$) influx in cultured endothelial cells from the rat aorta. **A**. Absolute values of the activity of $Na^+,K^+$-pump (ouabain-sensitive component of the rate of $^{86}Rb$-influx), NKCC (ouabain-resistant, bumetanide-sensitive component of the rate of $^{86}Rb$-influx) and passive permeability for $K^+$ (ouabain + bumetanide)-resistant 86Rb influx). **B**. Dose-dependent actions of NaHS on $Na^+/K^+$-pump (**1**), NKCC (**2**) and the rate (ouabain + bumetanide)-resistant $K^+$ ($^{86}Rb$) influx (**3**). The values obtained in the absence of NaHS were taken as 100%. Means and standard errors obtained in **5** independent experiments are shown. * - $p < 0.05$ compared to controls.

**Table 2**
Effect of 100 μM NaHS, bumetanide, TEA and nicardipine on the rate of $^{45}Ca$ influx in cultured smooth muscle cells.

| Additions, μM | $^{45}Ca$ influx, pmol/mg protein/5 min | |
|---|---|---|
| | $[K^+]_o = 5$ mM | $[K^+]_o = 30$ mM |
| 1. None (control) | 100 | 278 ± 23* |
| 2. NaHS, 100 | 110 ± 20 | 387 ± 25* |
| 3. NaHS, 100 + bumetanide, 10 | 101 ± 17 | 289 ± 22*,# |
| 4. NaHS, 100 + TEA, $10^4$ | 108 ± 19 | 367 ± 30* |
| 5. NaHS, 100 + nicardipine, 2 | 125 ± 21 | 104 ± 10# |

Means ± S.E. obtained in 5 independent experiments performed in quadruplicates are shown. The rate of $Ca^{2+}$ influx at $[K^+]_o$ = 5 mM and in the absence of any additions listed in the left column varied from 389 and 425 nmol / mg protein / 5 min was taken as 100%.

* - $p < 0.05$ compared to $^{45}Ca$ influx at $[K^+]_o$ = 5 mM.

# - $p < 0.05$ compared to control at $[K^+]_o$ = 30 mM in the presence of 100 μM NaHS ( there was no significant differences of $Ca^{2+}$ influx in the presence of additions at $[K^+]_o$ = 5 mM).

**Table 3**
Effect of 1000 μM NaHS, bumetanide, TEA and nicardipine on the rate of $^{45}Ca$ influx in cultured smooth muscle cells.

| Additions, μM | $^{45}Ca$ influx, pmol/mg protein/5 min | |
|---|---|---|
| | $[K^+]_o = 5$ mM | $[K^+]_o = 30$ mM |
| 1. None (control) | 100 | 278 ± 23* |
| 2. NaHS, 1000 | 106 ± 15 | 177 ± 18* |
| 3. NaHS, 1000 + bumetanide, 10 | 113 ± 21 | 170 ± 25* |
| 4. NaHS, 1000 + TEA, $10^4$ | 112 ± 16 | 277 ± 18*,# |
| 5. NaHS, 1000 + nicardipine, 2 | 88 ± 14 | 100 ± 12# |

Means ± S.E. obtained in 5 independent experiments performed in quadruplicates are shown. The rate of $Ca^{2+}$ influx at $[K^+]_o$ = 5 mM and in the absence of any additions listed in the left column varied from 389 and 425 nmol / mg protein / 5 min was taken as 100%.

* - $p < 0.05$ compared to $^{45}Ca$ influx at $[K^+]_o$ = 5 mM.

# $p < 0.05$ compared to control at $[K^+]_o$ = 30 mM, in the presence of 1000 μM NaHS ( there was no significant differences of $Ca^{2+}$ influx in the presence of additions at $[K^+]_o$ = 5 mM).

precontracted with norepinephrine [12]. Most studies reported blood plasma $H_2S$ concentration at levels up to $5 \times 10^{-5}$ M [13,31]. We found that aortic ring relaxation triggered by high doses of NaHS is abolished in the presence of TEA, a potent inhibitor of $K^+$ channels, thus indicating activation of these channels. This conclusion is also consistent with data obtained with specific inhibitors of $K_{ATP}$ and $K_{Ca}$ channels [1,15,39–43]. It is well-documented that activation of $K^+$ channels leads to SMC hyperpolarization and partial inactivation of voltage-gated $Ca^{2+}$ channels [44]. Indeed, treatment with $10^{-3}$ M NaHS resulted in suppression of $^{45}Ca$ influx evoked by modest depolarization (Table 1) that is consistent with dose-dependent inhibition by NaHS of L-type $Ca^{2+}$ channels obtained by patch clamp [45].

In contrast to inhibitory effects of NaHS, the mechanisms of activation of contractile responses by low doses of this compound remains poorly understood. Telezhkin and co- workers demonstrated the inhibitory effects of $H_2S$ on the α-subunit of big-conductance calcium-activated potassium ($BK_{Ca}$) channels in heterologously transfected HEK293 cells [24]. Likewise, $H_2S$ inhibited native $BK_{Ca}$ channels in type 1 glomus cells from the isolated mouse carotid body [46] and colonic longitudinal muscle and circular muscle strips [47]. H2S donor directly activated $BK_{Ca}$ currents in mouse coronary smooth muscle cells [48], piglet cerebral arterioles [49,50] and in endothelial cells from rat mesenteric arteries [51,52]. Guo and co-workers reported that $BK_{Ca}$ blockers inhibits $H_2S$-induced relaxation [49]. Our previous results didn't reveal any modulation of $H_2S$-induced contractions in the presence of $BK_{Ca}$ blocker charybdotoxin [53].

Our results demonstrate for the first time that contractile actions of low doses of NaHS is at least partially mediated by activation of $Na^+,K^+,2Cl^-$ - cotransport that, in turn, leads to elevation of intracellular chloride concentration, SMC depolarization and activation of voltage-gated $Ca^{2+}$ channels. This conclusion is supported by several observations listed below. *First*, contractions of aortic rings triggered by NaHS at concentrations less than $10^{-4}$ M were completely abolished in the presence of bumetanide (Fig. 2), a potent and selective inhibitor of $Na^+,K^+,2Cl^-$ cotransport. It might be also assumed that the lower increment of contraction by modest doses of NaHS seen in intact aorta is partially caused by diffusional problems of this compound to $Na^+,K^+,2Cl^-$ cotransporter localized in SMC sarcolemma. *Second*, low doses of NaHS increased activity of $Na^+,K^+,2Cl^-$ - cotransport in cultured SMC without any significant impact on other $K^+$ ($^{86}Rb$) transport systems (Fig. 4). *Third*, the contractile action of low doses of NaHS was increased in aortic rings subjected to modest depolarization the presence of 30 mM KCl as compared to depolarization at 60 mM KCl (Fig. 1). Previously, we reported that bumetanide inhibits contractions evoked by partial depolarization via elevation of intracellular concentration of $Cl^-$ and plasma membrane depolarization [17]. *Fourth*, the treatment with $10^{-4}$ M NaHS resulted in ~1.5-fold elevation of depolarization-induced $^{45}Ca$ influx that was suppressed by bumetanide and completely abolished in the presence of inhibitor of voltage-gated L-type $Ca^{2+}$ channel nicardipine (Table 1).

Previously, it was shown that hydralazine, a potent stimulator of $K^+,Cl^-$ cotransport, i.e. another carrier playing a key role in $[Cl^-]_i$ regulation, reduced tension in precontracted porcine aortic strips [54]. In mammalian erythrocytes $K^+,Cl^-$ cotransport is completely inhibited by 100 μM DIOA [55]. It should be noted that at this concentration DIOA exerts diverse side-effects on cultured SMC [56] and cannot be used to analyze the role of this carrier in contraction regulation.

Data obtained by Lim and co-workers suggest that vasoconstriction of NaHS-treated aortic rings involves inhibition of adenylyl cyclase / cAMP pathway [57]. We found that contractile actions of low doses of NaHS are increased in endothelium-denuded aortic strips as compared to intact ones (Fig. 2) thus suggesting that endothelium dysfunction promotes the $H_2S$-induced contraction. This conclusion is consistent with augmented contractile actions of low doses of NaHS after inhibition of nitric oxide-mediated signaling in intact human internal mammary artery pretreated with acetylcholine [15]. On the other hand, the NaHS-induced contraction of rat coronary artery was elevated following the removal of endothelium or the use of the nitric oxide synthase inhibitor L-NAME [37]. Considering these results, it should be noted that molecular mechanisms underlying the regulation of contractile responses of evoked by NaHS are species- and vascular bed-specific [1,7,58,59]. It is also important to underline that unlike the above-cited studies our experiments were performed in the absence of bicarbonate anions. Previously we reported that addition of 25 mM $NaHCO_3$ inhibits NKCC and attenuates the inhibitory action of bumetanide on contractions of mouse mesenteric artery evoked by 30 mM KCl and PE [35].

In contrast to SMC, we did not find any activation of NKCC in NaHS-treated endothelial cells (Fig. 5) expressing the same NKCC1 isoform of this carrier [60]. Thus, it might be assumed that $H_2S$-induced regulation of NKCC targets unknown intermediates of SMC-specific signaling rather than NKCC1 per se. We also revealed that NaHS sharply inhibits $Na^+,K^+$-pump in endothelial but not in SMC. Recently, we demonstrated that elevation of the $[Na^+]_i/[K^+]_i$ ratio in endothelial cells evoked by sustained $Na^+,K^+$-pump inhibition is accompanied by sharp elevation of cyclooxygenase COX-2 expression [61]. It is known that side-by-side with elevation of cGMP in SMC triggered by NO release, endothelium-dependent vasorelaxation is initiated by COX-mediated synthesis of prostacyclin $PGI_2$ [59,62,63]. The role of recently discovered $Na_i^+,K_i^+$-sensitive mechanism of excitation-transcription coupling [64] in vasorelaxation triggered by $Na^+,K^+$-pump inhibition and COX-2 transcription in endothelial cells treated with high doses of $H_2S$ remains unknown.

***In conclusion***, our results strongly suggest that excitatory actions of NaHS ($5x10^{-6}$ to $10^{-4}$ M) on vascular smooth muscle contraction are mediated via activation of $Na^+$,$K^+$,2$Cl^-$-cotransport and $Ca^{2+}$ influx through voltage-gated L-type $Ca^{2+}$ channels. As well, additional experiments should be performed to clarify the relative impact of $Na^+$,$K^+$-pump and other anion transporters in bimodal involvement of $H_2S$ in vascular tone regulation.

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## Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.09.010.

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