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Sodium Hydrosulfide Exerts a Transitional Attenuating Effect on Spermatozoa Migration *inVitro*

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Hydrogen sulfide (H_2S) has been shown to have a prominent role in the regulation of reproductive system function and fertility. The aim of the study was to assess the effect of a H_2S donor, sodium hydrosulfide (NaHS), on mouse sperm migration *in vitro*. Special plates with 4 corner wells filled with balanced salt solution (control) and various NaHS solutions in concentrations of 2.5 mmol/l, 5 mmol/l or 10 mmol/l were applied. Spermatozoa from each male mouse were injected (the experiment was repeated with ten BALB/c 5-month old males) into the central pocket, connected with the wells with ducts. After 1 h, 2 h and 4 h of incubation, the number of spermatozoa in each well was determined using Bürker's counting chambers. The number of spermatozoa in all corner wells were summed and the number of the cells in each well was treated as the percentage share of all the migrated spermatozoa. At the time points of 1 hour and 4 hours, no differences regarding chemotactic features of spermatozoa to the utilized solutions were observed. After two hours of incubation the attenuating effect of NaHS medium and high level solutions on spermatozoa migration was observed, but not for the low concentration mixture: $H(3, N = 40) = 9.65$, $P = 0.022$; control group vs 5 mmol/l NaHS solution: 36.0% vs 18.5%, $P = 0.023$; control group vs 10 mmol/l NaHS solution group: 36.0% vs 17.0%, $P = 0.011$. In conclusion, NaHS has a transitional attenuating effect on spermatozoa migration *in vitro*.

Key words: Sodium hydrosulfide, hydrogen sulfide, spermatozoa, cell migration, chemotaxis, *in vitro*.

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Chemotaxis is the phenomenon of gradient-directed, free migration of cells in the environment of a chemical compound. It was detected not only in lower animals but was also observed in mammals and humans (ŚLIWA 1989). Chemotaxis is one of the key factors in fertilization (EISENBACH & RALT 1992). Different particles, components of follicular fluid, act as chemoattractants. The most potent agents are hormones with progesterone at the forefront (ŚLIWA 1994; ŚLIWA 1995). Other constituents of follicular fluid including growth factors and heparin were also disclosed to exert chemotactic and chemokinetic actions on spermatozoa (RALT *et al.* 1994; ŚLIWA 1993, 2003; ŚLIWA & WILIŃSKI 1985). Moreover, a number of studies on mammals have shown that chemotaxis plays a pivotal role in other physiological and pathological processes including inflammation, wound healing, atherogenesis, metastasis or even nerve regeneration and is mediated by different

compounds (MUHEREMU *et al.* 2013; TERRANOVA *et al.* 1995; TURNER *et al.* 2014). Thus, numerous agents having a regulatory influence on cell physiology might participate in the ordering of spermatozoa swimming behavior. Hence, we hypothesize that hydrogen sulfide (H_2S, sulfane) is one of these agents. H_2S is a natural modulator of numerous physiological and pathological processes in mammals with multidirectional actions in cell biology (KIMURA 2011) and has been shown to have regulatory properties in leukocyte migration and trafficking (DAL-SECCO *et al.* 2008; ZHANG *et al.* 2007).

The confirmation of the participation of H_2S in spermatozoa chemotaxis would broaden the knowledge of reproductive physiology and might open fields for research on new methods of infertility treatment and enhance *in vitro* fertilization efficacy.

The aim of the study is to assess the effect of sodium hydrosulfide (NaHS), a H_2S donor, on mouse spermatozoa migration *in vitro*.

Material and Methods

The study has been approved by the I Local Ethics Committee of the Jagiellonian University (Kraków, Poland) with permission no. 62/2012 issued on 23 May 2012.

Ten BALB/c male mice (5-month old individuals) were killed by cervical dislocation. Sperm samples of every animal were collected from both vasa (ducti) deferentia and suspended in balanced salt solution (PBS). In order to separate the spermatozoa from the vas deferens plasma, the suspension was centrifuged at 300 G for 2 min. Subsequently, the medium was decanted and the sperm samples were resuspended in PBS. The procedure was performed 3 times altogether, since no significant effect of such preparation on sperm motility was recorded (MAKLER & JAKOBI 1981). Then, samples with at least 60% of normal-shaped spermatozoa showing forward motility were considered for further evaluation.

The study of spermatozoa migration was conducted by means of an authorial chemotactic chamber using a concentration gradient of the analyzed compound (ŚLIWA 1993). In a nontoxic plastic Perspex plate (sized 50 x 50 x 10 mm), 5 wells of 10 mm diameter and 15 mm depth were constructed. One was situated centrally and four were placed at the plate's corners. Corner wells were linked with the central pocket with ducts of 3 mm depth and 1 mm width. The basal well was filled with 500 μl of PBS with mouse spermatozoa. Corner pockets were filled with pure PBS (control group) or NaHS solutions of 2.5 mmol/l, 5 mmol/l or 10 mmol/l concentrations, respectively. The chamber was incubated in the ambient temperature. After 1, 2 and 4 hours of observation, the fluid of each corner compartment was sampled and subsequently the spermatozoa numbers were determined using Bürker's counting chambers. Since the amounts of sampled spermatozoa highly varied and the study group was small, the following system of analysis was used: the numbers of spermatozoa in all corner wells were summed (the total number of migrated spermatozoa) and the number of spermatozoa in each well were treated as the percentage share of all migrated spermatozoa.

The statistical analysis was performed with the Statistica 10 PL version (Statsoft, Tulsa, USA). Differences in spermatozoa numbers among control and all NaHS concentration wells after 1 h, 2 h and 4 h were assessed with Kruskal-Wallis one-way analysis of variance. The Mann-Whitney U test was utilized for comparisons between different pairs of groups. Statistical significance was considered when $P<0.05$.

Results

At the time points of one hour and four hours, no differences regarding chemotactic properties of the applied solutions were observed: $H(3, N = 40) = 3.56$, $P = 0.31$ and $H(3, N = 40) = 3,80$, $P = 0.28$, respectively. After two hours of the incubation, the attenuating effects of NaHS medium and high level solutions on spermatozoa migration were observed, but not for the low level mixture,: $H(3, N = 40) = 9.65$, $P = 0.022$; control group vs 2.5 mmol/l NaHS solution group: 36.0% vs 28.5%, $P = 0.24$; control group vs 5 mmol/l NaHS solution group: 36.0% vs 18.5%, $P = 0.023$; control group vs 2.5 mmol/l NaHS solution group: 36.0% vs 17.0%, $p = 0.011$ (Table 1, Fig. 1).

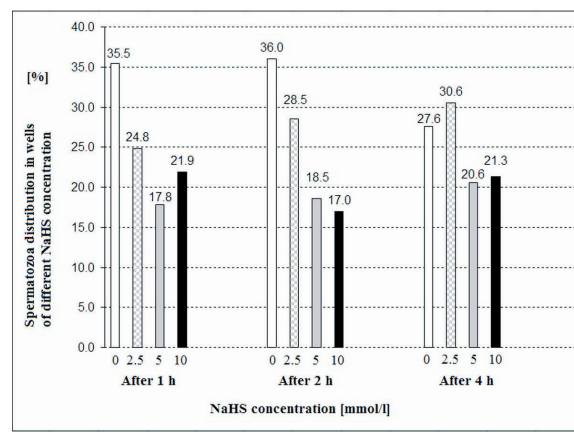

Fig. 1. Percentage share of spermatozoa numbers in each well of the control group and groups of different sodium hydrosulfide (NaHS) concentration solutions: 2.5 mmol/l, 5 mmol/l and 10 mmol/l (named control, 2.5, 5 and 10, respectively) at all time points of observation (1 h, 2 h and 4 h of incubation).

Table 1

Numbers of spermatozoa in Bürker's counting chambers in the control group and groups of different sodium hydrosulfide (NaHS) concentration solutions: 2.5 mmol/l, 5 mmol/l and 10 mmol/l (named control, 2.5, 5 and 10, respectively)

After 1 h					
Groups	Total No	control	2.5	5	10
1	4239000	3208000	758000	148000	125000
2	605000	136000	93750	156250	219000
3	3581000	2917000	195000	305000	164000
4	4335000	898000	742000	945000	1750000
5	1421000	359000	773000	164000	125000
6	358000	78000	70000	62000	148000
7	476000	62000	94000	211000	109000
8	1391000	680000	523000	94000	94000
9	952000	140000	414000	109000	289000
10	226000	70000	39000	62000	55000
After 2 h					
Groups	Total No	control	2.5	5	10
1	3426500	963500	703000	1489000	271000
2	496750	177000	109000	93750	117000
3	1332625	742000	117000	140625	333000
4	2211000	328000	430000	656000	797000
5	1515000	898000	430000	39000	148000
6	1031000	281000	289000	281000	180000
7	554000	234000	148000	102000	70000
8	1461000	586000	687000	102000	86000
9	1179000	109000	742000	78000	250000
10	147000	70000	31000	31000	15000
After 4 h					
Groups	Total No	control	2.5	5	10
1	4505000	1026000	1175000	1687000	617000
2	1269000	250000	175000	547000	297000
3	1921000	234000	578000	492000	617000
4	1191000	19000	78000	469000	625000
5	437000	180000	187000	23000	47000
6	555000	156000	250000	55000	94000
7	265000	133000	62000	31000	39000
8	1040000	352000	508000	125000	55000
9	984000	203000	500000	78000	203000
10	171000	78000	31000	23000	39000

Numbers of spermatozoa in Bürker's counting chambers in the control group and groups of different sodium hydrosulfide (NaHS) concentration solutions: 2.5 mmol/l, 5 mmol/l and 10 mmol/l (named control, 2.5, 5 and 10, respectively).

Discussion

The formation of H_2S in mammalian cells is catalyzed by cystathionine â-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST). H_2S is also a product of different non-enzymatic reactions and is present in tissues in the forms of free H_2S and within organized stores as cytoplasmatic bound sulfane sulfur and acid-labile sulfur (KIMURA 2011; WILIŃSKI *et al.* 2010). Its concentration in body fluids and tissues varies from <1 μmol/l to >600 μmol/l and can be much higher locally as H_2S is involved in autocrine/paracrine cell function regulation (LIM *et al.* 2013; WILIŃSKI *et al.* 2011a, 2011b).

The expression of H_2S-generating enzymes has been identified in male and female reproductive systems in different mammalian species (ZHU *et al.* 2011). CSE is localized in Sertoli cells and immaturate germ cells involving spermatogonia, while CBS activity was detected in Leydig cells, Sertoli cells and germ cells (OI *et al.* 2001). H_2S and non-organic sulfur compounds play a crucial role in the regulation of testis function and sperm maturation – sperm component stabilization, the acquisition of sperm motility and fertilization ability (SELIGMAN *et al.* 2005). Endogenous H_2S is pivotal for facilitating erectile function (LIAW *et al.* 2011).

CBS and CSE have been also detected in the uterus, ovary, follicular cells, vaginal, placental and fetal tissues (SRILATHA *et al.* 2009; ZHU *et al.* 2011). H_2S regulatory function is pivotal in oocyte maturation and in maintaining fertility (LIANG *et al.* 2007). CBS-deficiency in female mice led to uterine failure and infertility (GUZMÁN *et al.* 2006). Moreover, H_2S was shown to attenuate the response to oxytocin, thereby delaying parturition (HAYDEN *et al.* 1989).

Studies have shown that H_2S affects migration of various types of cells under different conditions. In the experiment of TAO *et al.* (2012), NaHS promoted vascular endothelial cell migration under normoxic conditions. H_2S increased the level of hypoxia-inducible factor 1α (HIF-1α) in a dose- and time-dependent manner and decreased reactive oxygen species (ROS). PUPO *et al.* (2011) showed that NaHS enhances cytosolic calcium and activates the migration of human breast carcinoma cells (B-TECs), but not 'normal' human microvascular endothelial cells (HMVECs), and this tumor proangiogenic signaling is triggered by vascular endothelial growth factor (VEGF). SPILLER *et al.* (2010) demonstrated that H_2S improves leukocyte rolling/adhesion in the mesenteric microcirculation as well as neutrophile migration in the animal model of sepsis based on cecal ligation. H_2S prevented the down-regulation of chemokine receptor CXCR2 and l-selectin and the up-regulation of CD11b and G protein-coupled receptor kinase 2 in neutrophiles by an ATP-sensitive K^+ channel-dependent mechanism.

In our study NaHS exerted a negative chemotactic effect on spermatozoa *in vitro*. A similar phenomenon was observed when polymorphonuclear leukocytes (PMN) were incubated in a 1-2 mmol/l H_2S solution (PERSSON *et al.* 1993). Analogically, in the experiment of Fang and colleagues, H_2S decreased human lung fibroblast (MRC5) migration stimulated by fetal bovine serum (FBS) and basic fibroblast growth factor (bFGF) and their proliferation. Interestingly, migration was suppressed directly – not mediated by the ATP-dependent K^+ channel – and was at least partially attributed to a decrease in extracellular signal-regulated kinase (ERK 1/2) phosphorylation (FANG *et al.* 2009).

Chemical attraction to sperm was initially reported in compounds that have membrane receptors such as hormones but later it became obvious that it involves agents capable of influencing cell metabolism or spermatozoa motility (ŚLIWA 1994). H_2S uses membrane the ATP-dependent K^+ channel as the first signaling step. It easily permeates cell membranes and has multiple intracellular actions using i.a. sulfhydration of proteins including kinases as a potent signaling measure (HANCOCK & WHITEMAN 2014). H_2S also penetrates mitochondria and in higher concentrations causes transitional inhibition of cytochrome oxidase (mitochondrial respiratory chain), what could explain the course of our experiment – the passing negative chemotactic effect, but the share of other mechanisms – including alteration of protein functions associated with spermatozoa movement - should also be considered (BOUILLAUD *et al.* 2013). Our study proves that H_2S affects spermatozoa swimming behavior. Its effects should be estimated in further research since chemotactic and chemokinetic effects result from a complex interplay of numerous compounds present in the body.

As a study limitation, the lack of a spermatozoa kinesis assessment needs to be emphasized. In conclusion NaHS, a H_2S donor, exerts a transitional attenuating effect on spermatozoa migration *in vitro*.

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