Research Article

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Effects of arginine vasopressin on migration and respiratory burst activity in human leukocytes

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Abstract: Arginine vasopressin can bind to high-affinity vasopressin V1a receptors in human leukocytes. This study aims to investigate the effects of arginine vasopressin on migration and chemotaxis of neutrophils and oxygen free radical release by human leukocytes. Neutrophils and monocytes were obtained from peripheral blood samples of ten healthy volunteers. Leukocyte migration was microscopically assessed in a modified 48-blind well microchemotaxis chamber, and respiratory burst activity was estimated using 2',7'-dichlorofluorescin diacetate in descending concentrations of arginine vasopressin. Arginine vasopressin stimulates migration of monocytes and neutrophils depending on concentration and on interaction with other chemoattractants. The strongest chemotactic responses of monocytes to arginine vasopressin were observed in the micro and nanomolar range and in the nanomolar range for neutrophils ($p<0.001$). Pre-incubation of leukocytes with arginine vasopressin decreased migration of leukocytes in a dose-dependent manner. Arginine vasopressin did not stimulate release of oxygen free radicals by neutrophils. Arginine vasopressin stimulates in a dose-dependent manner the migration of monocytes and neutrophils. However, pre-incubation of leukocytes with arginine vasopressin decreased the migratory response of monocytes and neutrophils to other chemoattractants. These findings may be of importance in the treatment regimen of patients with septic shock.

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1 Introduction

Arginine vasopressin (AVP) is a nine-amino-acid peptide hormone synthesized in the hypothalamus and stored in vesicles at the posterior pituitary [1]. Its main physiologic functions are re-absorption of water in the renal collecting ducts, and vasoconstriction [2]. In the anterior pituitary, AVP acts *via* V1b receptors [3]. *Via* the hypothalamus-pituitary-adrenal axis, it influences the regulation of adrenocorticotropic hormone (ACTH) secretion by the pituitary gland and as a consequence, release of corticosteroid by the adrenal glands [4]. AVP acts as a chemoattractant for small cell lung carcinoma cells and possibly for monocytes similar to bombesin [5]. Interestingly, in circulating blood cells, AVP almost exclusively binds to mononuclear phagocytes and much less to lymphocytes and polymorphonuclear leukocytes [6].

AVP has direct, high-affinity vasopressin V1a receptor-mediated pro- and anti-inflammatory effects on human leukocytes [4, 7]. *In vitro*, AVP induces production of interferon-gamma (IFN-γ) [8] and enhances lymphocyte response. Furthermore, AVP has immunomodulatory properties jointly with other vasoactive catecholamines [9, 10] and enhances macrophage phagocytosis. AVP-induced rapid release of oxygen radical and hydrogen peroxide (respiratory burst) degrades internalized particles, and in combination with hypochlorite, it directly destroys bacteria. Phagocytosis and subsequent respiratory burst are integral components of the immune response. In immune-deprived intensive care patients with systemic inflammation and septic shock, administration of AVP is frequently the mainstay of treatment.

We investigated the effects of AVP on migration and chemotaxis of and oxygen free radical release by human

leukocytes *in vitro*. Results of our study may provide new insights into endogenous inhibition of inflammation and elucidate the potential benefits of AVP administration in patients with septic shock.

2 Methods

2.1 Study design

An experimental study was performed in the medical laboratories of the university hospital of Innsbruck. This study was approved by the Ethics Committee of the Medical University Innsbruck. Written informed consent was obtained from all participants prior to inclusion in the study. Participation in the study was voluntary and based on the understanding that results will be published in scientific journals.

Neutrophils and monocytes were obtained from peripheral blood samples of ten healthy volunteers (10 mL samples, each anti-coagulated with 1.6 mg EDTA/mL blood). Neutrophils were prepared after lymphoprep density gradient centrifugation by dextran sedimentation followed by hypotonic lysis of contaminating erythrocytes with potassium chloride solutions as previously described [11]. After lymphoprep density gradient centrifugation, cells were collected and washed three times with normal saline. Biocoll (15 mL) separating solution was carefully covered with the blood and centrifuged at 300x*g* for 20 min. The peripheral blood mononuclear cell (PBMC) layer was carefully obtained and again centrifuged for 10 min at 300x*g*. The resulting cell pellet was resuspended in a standard solution consisting of RPMI1640 enriched with 0.5 % BSA. After determining of the amount of PBMCs, $CD14^+$ monocytes were isolated from PBMCs with magnetic cell sorting according to the manufacturer's protocol (Miltenyi Biotec).

2.2 Chemical substances

Bovine albumin serum (BSA) was from Sigma Chemical Corp (St. Louis, MO). For density centrifugation, Biocoll from Biochrome AG (Berlin, Germany) was used. The MACS Monocyte isolation kit II was from Miltenyi Biotec® (Bergisch Gladbach, Germany). RPMI 1640 with phenol red was from Biological industries (Kibbutz Beit Haemek, Israel). The nitrocellulose filter (5 µm pore size) was from Sartorius AG (Goettingen, Germany). Hank's balanced salt solution without calcium and magnesium was purchased from GIBCO BRL, Life Technologies (Vienna, Austria) and phosphate buffered saline (PBS) was from PAA (Linz, Austria). All other reagents not further specified were obtained from Sigma Chemical Corp (St. Louis, MO). All stock solutions were stored at -20°C. Vasopressin (BCN Peptides; Barcelona, Spain) was stored in PBS at a concentration of 10^{-3} M at -20 °C and diluted in assay medium for the experiments.

2.3 Chemotaxis experiments

Migration of leukocytes was measured using a modified 48-blind well microchemotaxis chamber (Neuro Probe, Cabin John, MD) equipped with 5-µm pore sized nitrocellulose filters for neutrophil or monocyte chemotaxis [17]. After washing, 50 µL of cell suspension (1 x 10^6 cells/mL) were put into the upper compartment of the chemotaxis chamber, and cells were allowed to migrate for 25 min (neutrophils) and 45 min (monocytes) toward different concentration gradients of the soluble chemoattractants N-formyl-Met-Leu-Phe (fMLP) or interleukin 8 and vasopressin in the lower wells. Fresh culture medium served as control substance. Chemotactic responses were tested in the range 10^{-5} to 10^{-15} M. After these migration periods, the filters were dehydrated, fixed, and stained with hematoxylin and eosin. Migration depth was quantified by microscopy, measuring the distance (µm) from the surface of the filter to the front line of the leading three cells. To investigate deactivation of migration by pre-treatment, cells were incubated for 30 min with different concentrations of vasopressin and chemoattractants and washed twice before testing for chemotaxis.

2.4 Respiratory burst activity of neutrophils

PMN were placed in a humidified incubator (37°C, 5% CO2) for various time periods with medium (basal activity) or triggering agents (stimulated activity): 1 µM N-formyl-Met-Leu-Phe (fMLP; Sigma Aldrich, Munich, Germany) or 324 nM phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, Munich, Germany). Respiratory burst activity was measured using H2DCFDA as a fluorochrome. The assay is based on the oxidation of non-fluorescent H2DCFDA to highly fluorescent 2'-7' dichlorofluorescein (DCF), both intracellularly and extracellularly [13]. Fluorescent activity was determined at 485 ± 20 nm excitation and 530 ± 25 nm emission wavelengths using a multiwell plate reader (Tecan Infinite M200)). Neutrophil respiratory burst activity was detected by an assay using 2',7'dichlor-

ofluorescin diacetate (DCFH-DA), as described previously [12]. This assay is based on the oxidation of nonfluorescent DCFH-DA to the highly fluorescent 2',7'-dichlorofluorescein (DCF) both intracellularly and extracellularly [14]. Neutrophils were primed for 30 min at 37°C (5% CO_2 atmosphere) with various concentrations of vasopressin, washed twice, and resuspended in Hanks' balanced salt solution (HBSS). Thereafter, 100 µL/well (96-well plate) of 2 x 10^5 neutrophils were immersed at 37°C in a 10-µml/L solution of DCFH-DA in phenol red-free HBSS containing 1 µmol/L N-formyl-Met-Leu-Phe (fMLP, as a triggering agent) or medium. The plates were covered with lids and placed in a humidified incubator for various time periods. Fluorescence activity was determined at 485 ± 25 nm excitation and 530 ± 25 nm emission wavelengths using a multi-plate reader (Tecan Infinite M200). Readings were taken every 10 min for 1 h. Oxygen free radical release was expressed as fluorescence arbitrary unit.

2.5 Definitions

"Chemotaxis index" defines the ratio between the distance of directed and undirected migration. Leukocyte chemotaxis describes the movement of leukocytes toward sites of inflammation directed by extracellular gradients of diffusible chemicals [14]. In contrast, chemokinesis describes the non-vectorial movement of cells prompted by chemicals [15]. Checkerboard analysis can display alterations of speed, frequency and direction of migration. In this study, we used checkerboard dilution assay as a type of Boyden chamber assay for leukocyte chemokinesis and chemotaxis [16].

2.6 Statistical analysis

Non-parametric test was used for calculations of related samples. Data were expressed as mean and SEM. Means were compared by Mann-Whitney U test or paired Student's *t*-test after Kruskal-Wallis analysis of variance. Analyses were performed using GraphPad Prism 7 (La Jolla, CA) or SPSS 16.0 software (SPSS Inc., Chicago, IL).

3 Results

A total of ten blood samples were investigated in 5 examinations.

3.1 Chemotactic index

Following 25 min incubation of neutrophils against fMLP and different concentrations of AVP, the mean migration depth was 86.08±14.56 µm. The chemotactic index for neutrophils ranged from 110.2 to 123.3% (Fig.1A). After 45 min incubation of monocytes, the mean migration depth was 116.0 $\pm$ 14.1 µm. Data expressed as chemotactic index ranged from 110.8% to 116.8% (Fig.1B). Following 30 min pre-incubation with, various concentrations of AVP and migration against fMLP at 37°C the mean migration depth for neutrophils was 69.4 $\pm$ 9.4 µm. The chemotactic index for neutrophils ranged from 101.6% to 127.9 % (Fig.2A). The mean migration depth for monocytes was 70.8 $\pm$ 11.6 µm; the chemotactic index ranged between 99.86% and 127.77% (Fig.2B). Priming of the leukocytes with different concentrations of AVP and migration against AVP 10-9 M resulted in deactivation of the migratory response (Fig. 3A and 3B).

The strongest chemotactic responses to AVP were observed in the micro- and nanomolar range (10^{-6} to 10^{-9}) for monocytes and in the nanomolar range (10^{-9}) for neutrophils ($p<0.001$). Migration significantly differed between pre-incubated and not pre-incubated neutrophils at low dilutions (10^{-5} $p=0.023$; 10^{-7} $p=0.012$) and monocytes at high dilution (10^{-15} $p=0.007$). With declining AVP concentration, the blocking effect of AVP on migration diminished. Checker-board analysis revealed that neutrophils (Table 1 A) only migrate in a chemotactic manner, but AVP revealed chemotactic and chemokinetic properties of monocytes (Table 1B).

3.2 Respiratory burst activity

Following 10 min priming at 37°C with various concentrations of AVP, washing and re-suspension with HBSS and incubation with DCFH-DA, no release of oxygen free radicals was observed in neutrophils (Fig. 4).

4 Discussion

In this preliminary investigation, we observed that pre-incubation of leukocytes with AVP decreased the migratory response of immune cells to other chemoattractants and homologous deactivation of the migratory response. Checkerboard analysis showed that AVP in varying concentrations had chemotactic influence on neutrophils and chemotactic and chemokinetic influence on monocytes.

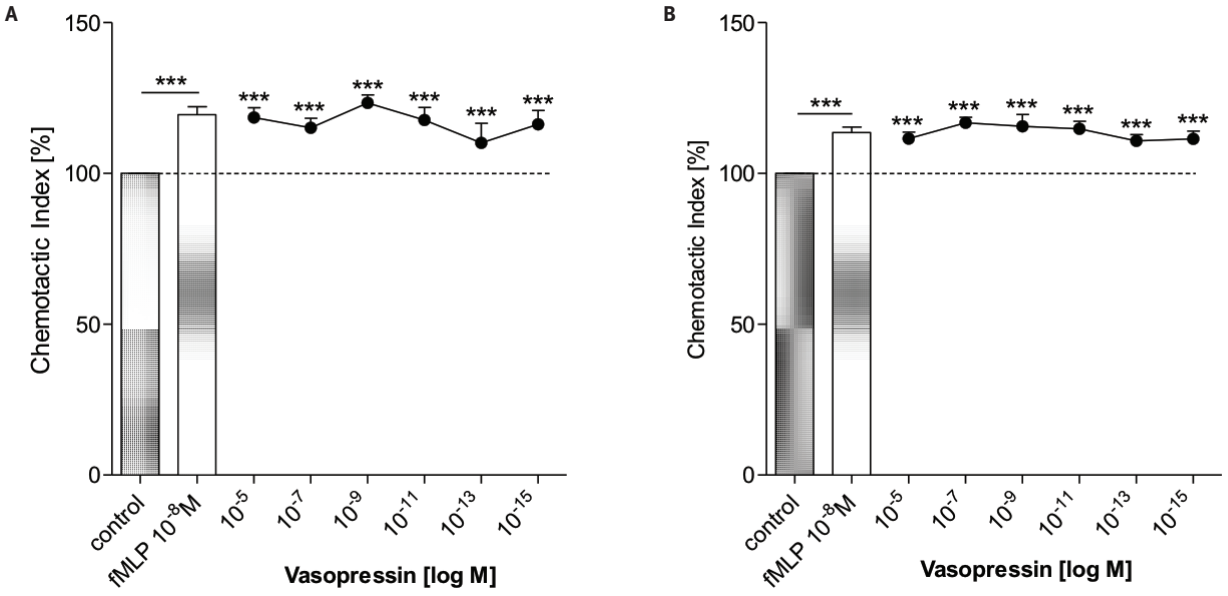

Figure 1: Direct chemotaxis against different concentrations of AVP

PMNs (A) and monocytes (B) remained untreated and were allowed to migrate towards medium (control), fMLP [10^{-8} M] and different concentrations of AVP. After fixing and staining the filters, migration depth was evaluated microscopically. Data are expressed as mean ± SD of the Chemotactic Index, which is the ratio between the distance that cells migrated towards the tested substances and that toward the control medium. Statistical analyses: Mann-Whitney U test

[**, p<0.01, ***, p<0.0001]

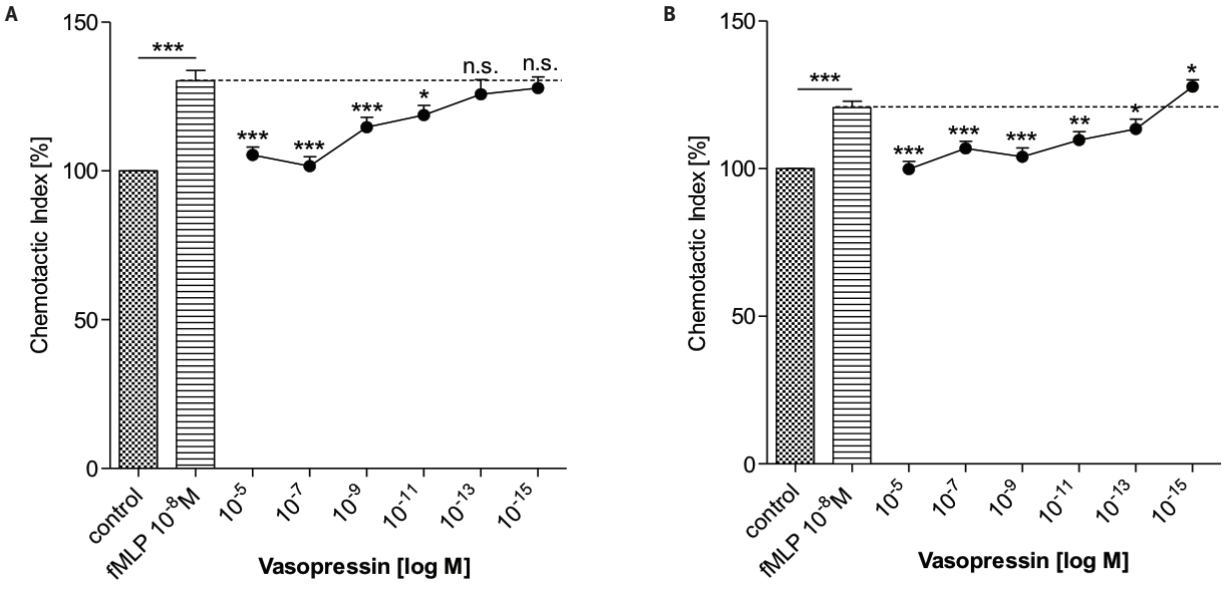

Figure 2: Priming cells with different concentrations of AVP

PMNs (A) and monocytes (B) were pre-incubated with different concentrations of AVP for 30 min and migrated against fMLP [10-8 M]. Migration towards medium served as the negative control, as positive control, migration against fMLP [10-8 M]. Statistical analyses: Mann-Whitney U test [n.s., not significant, *, p<0.05, **, p<0.01, ***, p<0.0001]

From the results of our preliminary investigation we cannot tell whether the chemotactic effect of AVP is receptor-independent or not. We assume that the receptor will be similar to the AVP V1-receptor in human peripheral blood mononuclear cells [7]. To identify both, receptor and second messenger will be focus of our future research.

Shock related to various illnesses, including sepsis and hemorrhage, is a known complication and a cause

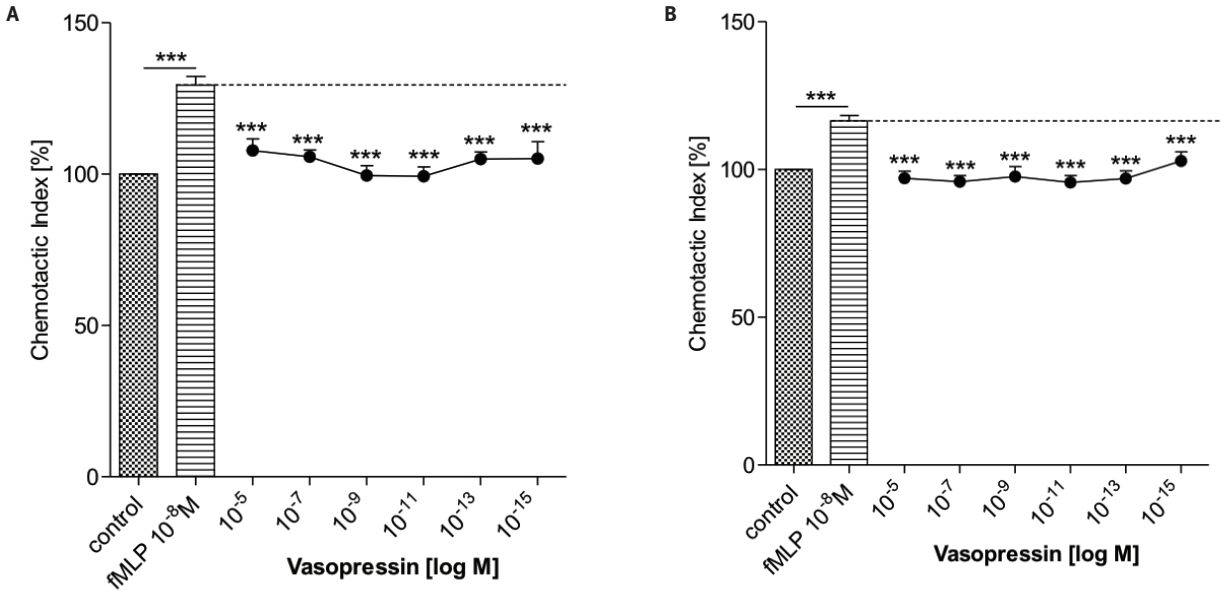

Figure 3: Priming cells with different concentrations of AVP

PMNs (A) and monocytes (B) were pre-incubated with different concentrations of AVP for 30 min and migrated against AVP [10-9 M]. Migration towards medium served as the negative control, as positive control, migration against fMLP [10-8 M]. Statistical analyses: Mann-Whitney U test [n.s., not significant, *, p<0.05, **, p<0.01, ***, p<0.0001]

Table 1: Checkerboard analysis in response to AVP

A)		Vasopressin [log M]. upper chamber		
	Medium	**10-Jul**	**10-Sep**	**10-Nov**
Vasopressin				
[log M] lower chamber				
Medium	1,000±0,000	0,953±0,157	1,098±0,051*	1,154±0,134
10-Jul	1,105±0,058*	0,935±0,128	1,022±0,099	1,088±0,081
10-Sep	0,982±0,117	0,935±0,166	1,073±0,112	0,978±0,089
10-Nov	1,015±0,086	1,012±0,144	1,049±0,055	1,046±0,095
B)				
		Vasopressin [log M]. upper chamber		
	Medium	**10-Jul**	**10-Sep**	**10-Nov**
Vasopressin				
[log M] lower chamber				
Medium	1,000±0,000	1,160±0,052**	1,170±0,112**	1,025±0,074
10-Jul	1,115±0,106**	1,072±0,096*	1,124±0,096**	0,981±0,080
10-Sep	1,137±0,066**	1,064±0,097	1,094±0,093**	1,077±0,074
10-Nov	1,160±0,103*	1,121±0,092*	1,081±0,087*	1,024±0,117

PMNs (A) and monocytes (B) migrated in response to different concentrations of AVP. The upper wells were filled with cells supplemented with various concentrations of AVP. The lower wells were filled with medium (control), or different concentrations of AVP. Statistical analyses: Mann-Whitney U test [n.s., not significant, *, p<0.05, **, p<0.01]

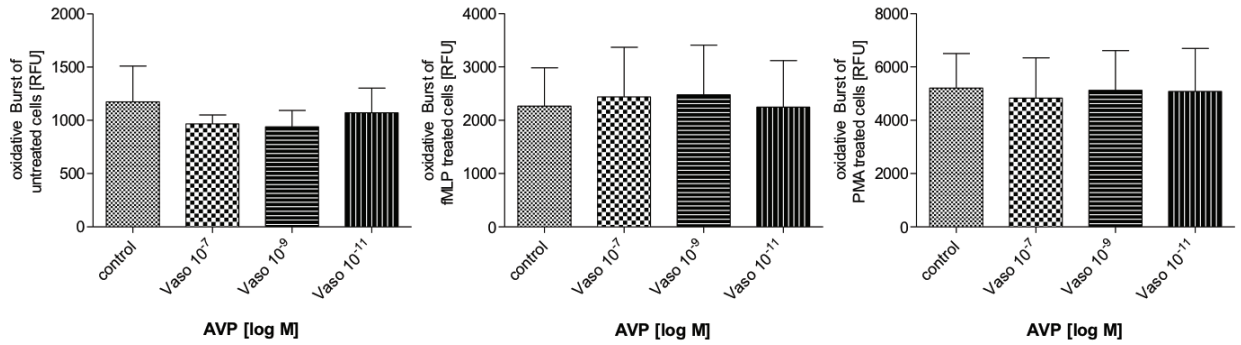

Figure 4: In vitro effects of AVP on oxidative burst

Oxidative burst of (A) unstimulated, (B) N-formyl-Met-Leu-Phe (fMLP) and (C) phorbol 12-myristate 13-acetate (PMA)-stimulated polymorphonuclear leukocytes (PMN); PMNs were incubated with various concentrations of AVP ranging from 10-7 to 10-11 M or control; Graph shows mean ± standard deviation; n = 5; RFU, relative fluorescence units.

of death of patients in intensive care. It is of paramount importance to extend our knowledge regarding immunomodulatory effects of vasopressors, in particular vasopressin, as used in the treatment of shock. Catecholamines, however, diminished the migratory response of immune cells as well [17], and procalcitonin, a marker and mediator of the immune system, was reported to reduce chemotaxis in human monocytes [11]. In our opinion, stress hormones and mediators of the acute-phase response, such as procalcitonin, are immunosuppressive since they decrease migration of leukocytes when administered in high doses for prolonged periods, and thus increase mortality.

In vasopressin-deficient rats with permanently decreased number of blood leukocytes and reduced macrophage activity, administration of vasopressin resulted in enhanced phagocytosis of peritoneal macrophages [18,19]. On the one hand, increased vasopressin production may indicate developing chronic inflammatory disease [9, 20]. On the other hand, vasopressin may limit immune response as illustrated by vasopressin-induced reduction in TNF-mediated inflammation in the rat cremaster microcirculation [21]. In bacterial urinary tract infection, vasopressin even down-regulates chemokine secretion, thereby limiting host defense [22].

Doherty et al. reported that vasopressin does not stimulate migration of polymorphonuclear leukocytes [23]. The findings of our study show more precisely that there is an AVP dose-dependent induction of chemotaxis in the presence of another chemoattractant depending on its concentration. However, when neutrophils were pre-incubated with AVP, there was significant reduction of migration in the presence of another chemoattractant.

AVP failed to enhance superoxide anion release of macrophages in comparison to substance P [24]. This is consistent with our findings. Depending on the results of our preliminary investigation, further experimental and clinical research is planned to assess the influence of vasopressin on leukocyte function in patients with SIRS and septic shock. AVP stimulates dose-dependent migration of monocytes and neutrophils. However, preincubation of leukocytes with AVP decreased the migratory response to other chemoattractants. This is in line with the known effect of catecholamine on the migratory response of human leukocytes [18]. Procalcitonin, a marker and mediator of the immune system, has the same effect on human monocytes [12].

In our opinion, stress hormones and mediators of the acute-phase response, such as procalcitonin, are immunosuppressive since they decrease the migration of leukocytes if their serum levels rise substantially (the serum levels of procalcitonin rise 300- to-500-fold in shock or sepsis) or if they are given in non-physiological doses or over a long period of time such as administration of AVP during resuscitation and therapy of septic shock.

5 Limitations

The disadvantage of our investigation method with blind well chemotaxis chamber was the time-consuming and labour-intensive nature of the isolation technique and processing and the microscopic evaluation. The determination and interpretation of results depended on the experience of the observer. The observer was not informed about the hypotheses of the study and followed clearly defined investigation standards. As all investigations were performed by only one person there was at least no inter-observer variability in the readings.

6 Conclusion

The preliminary results of our study show that pre-incubation of leukocytes with AVP decreased their migratory response to other chemoattractants. The strongest chemotactic responses to AVP were observed in the micro- and nanomolar range for monocytes and in the nanomolar range for neutrophils. AVP did not stimulate the release of oxygen free radicals in neutrophils.

Contributors: FJW had the principal idea of the study, searched for and selected the references, prepared the subsequent versions. KW made the experimental work, analyzed the results and contributed to the first draft. FJW and KW are both the first authors. MST wrote the proposal of the study and contributed to the experimental work. MJ, CK discussed and supported the experimental design and work. WL helped in the analysis of the results and prepared the first draft.

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Conflict of interest statement: Authors state no conflict of interest.

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