Vet. Res. 33 (2002) 71–82
© INRA, EDP Sciences, 2002
DOI: 10.1051/vetres:2001007

Original article

Effect of 1-24ACTH administration on sheep blood granulocyte functions

Saverio PALTRINIERI*, Simona PANELLI, Stefano COMAZZI, Paola SARTORELLI

Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Sezione di Patologia Generale Veterinaria e Parassitologia, via Celoria 10, 20133 Milano, Italy

(Received 8 March 2001; accepted 27 August 2001)

Abstract – The objective of the present study was to determine the efficiency of blood neutrophils (PMN) taken from sheep during acute stress. Ten healthy Charolle sheep were sampled before treatment (T0) and 1 (T1), 2 (T2), 24 (T24) and 48 (T48) hours after 1-24ACTH administration. Ten sheep serving as the controls were sampled at the same time intervals, using saline solution instead of 1-24ACTH. At each time sampling, rectal temperature, heart rate, cortisol, glucose, non-esterified fatty acids (NEFA), total and differential leukocyte counts were evaluated. PMN were isolated after centrifugation of whole blood and hypotonic lysis of RBC. Chemotaxis was evaluated on a modified Boyden chamber using a nitrate cellulose filter and both Zymosan activated serum (ZAS) and interleukin-8 (IL-8) as chemoattractants. Phagocytosis was measured using both non-opsonized latex beads and fluoresceinated yeasts opsonized with homologous serum. Superoxide (O_2^-) production was evaluated by measuring superoxide dismutase-inhibitable reduction of ferricytochrome C, and adherence by a colorimetric assay of acid phosphatase activity of adherent cells. The administration of 1-24ACTH induced an acute stress reaction, indicated by the presence of clinical, biochemical and hematological changes. Adherence significantly increased from T0 to T2 in treated sheep. This might be responsible for the depression of non-specific immunity in stressed animals. Studies using stressors other than 1-24 ACTH are needed to verify the influence of other components of the stress reaction on PMN functions.

sheep / PMN / ACTH / stress / phagocytosis

Résumé – Effets de l'administration de 1-24ACTH sur la fonction des granulocytes sanguins de brebis. L'objectif de cette étude a été la détermination de l'efficacité des neutrophiles (PMN) prélevés à des brebis pendant un stress aigu. Pour cela, nous avons effectué des prélèvements de sang à 10 brebis Charolle saines avant d'administrer du 1-24ACTH (T0) et, de nouveau, 1 (T1), 2 (T2),

*Correspondence and reprints
Tel.: (39) 02 58358103; fax: (39) 02 58358095; e-mail: savepal@mailserver.unimi.it

24 (T24), 48 (T48) heures après ce traitement. Comme contrôle nous avons fait des prélèvements de sang à 10 brebis auxquelles nous avions administré de la solution saline au lieu de 1-24ACTH. A l'occasion de chaque prélèvement nous avons déterminé la température, la fréquence cardiaque et les niveaux sanguins de cortisol, glucose, acides gras non-esterifiés, des leucocytes totaux et différenciés. Nous avons isolé les PMN après centrifugation du sang et lyse hypotonique des hématies. Afin de vérifier l'activité chimiotactique des PMN, nous avons utilisé une chambre de Boyden modifiée ainsi que du sérum activé par le Zymosan (ZAS) et de l'interleukine-8 (IL-8) comme chimioattractants. Afin de mesurer la phagocytose, nous avons utilisé aussi bien des particules de latex non-opsonisées que de la levure opsonisée avec du sérum homologue marqué à la fluorescéine. Ensuite, nous avons quantifié la production d'anion superoxyde en mesurant la réduction (inhibée par l'enzyme superoxyde dismutase) du ferricytochrome C, et l'adhérence (par un essai colorimétrique qui se base sur l'activité de phosphatase acide des cellules adhérentes). L'administration de 1-24ACTH provoque un état de stress aigu, comme l'indique la présence d'altérations cliniques biochimiques et hématologiques. L'administration de 1-24ACTH provoque aussi une augmentation de l'adhérence de T0 à T2. Cette variation est peut-être responsable de la diminution de l'immunité non-spécifique chez les animaux sujets au stress. Afin de vérifier l'influence d'autres composants de la réaction du stress sur la fonction des PMN, il est nécessaire d'utiliser des agents stressants différents de 1-24ACTH.

mouton / PMN / ACTH / stress / phagocytose

1. INTRODUCTION

Adverse environmental conditions increase the prevalence of infectious diseases in animals. In ruminant intensive husbandry, several situations such as warm or cold environments, early weaning, crowding, grouping of animals of different origins, noise and transport, induce a decrease in humoral and cellular immunity [13].

All these changes can be a consequence of stress. Stressors stimulate the hypothalamic production of the Corticotropic Releasing Hormone (CRH) that in-turn induces pituitary ACTH production and secretion. Adrenal glands respond to ACTH by producing glucocorticoids. These molecules in ruminants induce many biochemical and hematological changes [14, 18]. In particular, ACTH administration induces neutrophilic leukocytosis with right shift, lymphopenia and eosinopenia. These changes are known as stress leukogram, with differences depending on the species, stress type and duration [9]. Pathophysiologic doses of ACTH induce greater changes than the therapeutic ones [7]. Chronic stress is characterized by minor changes in the leukogram [9].

Apart from these quantitative changes, functional impairment of leukocytes has been reported during stress. Data regarding phagocyte functions in spontaneous or experimentally induced stress, are often contradictory and depend on the type of stressor used and on the step of the phagocytic process studied [1, 7, 8, 12, 17–19, 21]. However the different steps of the phagocytic process have not yet been simultaneously evaluated.

The aim of the present work is to study the influence of acute stress, induced in sheep by the inoculation of 1-24ACTH (the biologically active fraction of the ACTH molecule), in different functional activities of circulating PMN.

2. MATERIALS AND METHODS

2.1. Sampling

Blood (40 mL in tubes containing EDTA, 10 mL in tubes without anticoagulant) was taken from the jugular vein of 20 clinically healthy, 5 years old, non lactating female Charolle sheep, which were kept in the University barn, fed with hay

and concentrated feed (0.5 kg per day) and water ad libitum. The sheep received anti-tetanic prophylaxis (Covexin 8, Shering-Plough, Union, NJ, USA) and were dewormed using 10 mg/kg of albendazole (Valbazen, Pfizer Animal Health, Exton, PA, USA). The study started two weeks after a 30-day period of acclimatization to the new habitat during which the sheep were subjected to repeated blood sampling (10 mL once every two days), to avoid stress due to the new habitat, handling and blood sample collection.

The sheep were randomly divided into two groups (10 sheep per group) and, based on the results of previous work using the same stressor [22], the experimental protocol was as follows:

T0 = basal sampling (8.30 AM, before feeding) and administration of 6.5 μg/kg, IM in the hind quarter, of 1-24ACTH (Synachten, Novartis Farma, Origgio, VA, Italy) or of 1 mL of saline solution (controls);

T1 = 1 h after 1-24 ACTH or saline administration;

T2 = 2 h after 1-24 ACTH or saline administration;

T24 = 24 h after 1-24 ACTH or saline administration;

T48 = 48 h after 1-24 ACTH or saline administration.

At each sampling time rectal temperature and heart rate were recorded.

2.2. Hematology and serum chemistry

A complete blood cell count was carried out on each blood sample collected in EDTA just after the sampling using an automatic blood cell counter (Hemat 8, SEAC S.R.L., Calenzano, FI, Italy). The differential leukocyte count was evaluated on May Grünwald-Giemsa stained smears by counting 200 cells.

Blood in the tubes without an anticoagulant was centrifuged (15 min, 1000 × *g*) just after withdrawal. The serum was removed, stored at –20 °C and used to determine cortisol, glucose and non-esterified fatty acids (NEFA) within one month. Cortisol was measured using a commercially available kit for Radioimmuno assay determination (Ria-cortctk-125, Sorin Biomedica, Saluggia, VC, Italy) validated for the sheep in preliminary tests. Serum glucose was determined using a spectrophotometer (Jasco V530, Jasco Corporation, Tokio, Japan) with the hexokinase-UV method [25] (Hospitex diagnostics, Firenze, Italy), and NEFA with an enzymatic-colorimetric method (Boheringer Mannheim, Mannheim, Germany).

2.3. PMN isolation

PMN were isolated according to the procedure suggested by Carlson and Kaneko [3], widely used for ruminant PMN isolation [3, 19, 21], using plastic tubes to avoid PMN adhesion to glass surfaces: blood from the EDTA coated tubes was divided into 4 aliquots of 10 mL, and after centrifugation (15 min, 1 000 × *g*), plasma and the upper layer (3 mm) of RBC were removed: a hypotonic lysis of RBC was carried out by adding 20 mL of distilled water to each tube. After gently mixing (30 s), a hypertonic NaCl solution (2.7 g/dL in phosphate buffer 0.0132 M, pH 7.2) was added to restore isotonicity. After centrifugation (10 min, 200 × *g*), the supernatant was removed. This lysis was then repeated as suggested by Buchta [2] for ovine RBC. The cell pellets were then transferred to a single tube, washed twice with 40 mL of phosphate buffered saline (PBS), centrifuged (10 min, 200 × *g*) and resuspended in 1 mL of Hanks balanced salt solution (HBSS). A cell count in a Bürker chamber, a trypan blue vitality test [15] and a differential leukocyte count on slides prepared by cytocentrifugation (5 min, 50 × *g*) in a sedimentation chamber (Neuroprobe, Cabin John, MD, USA) and stained with May

Grünwald-Giemsa were then carried out. The PMN recovery rate was calculated as follows: (total number of PMN in the cell suspension/total number of PMN in 40 mL of blood employed) × 100.

The cell suspension was then diluted in HBSS to a final dilution of 2×10^7 cells/mL.

2.4. Chemotaxis

The dilution of the cells was adjusted to 2×10^6 cells/mL in HBSS with 20 mmol/L HEPES (Sigma Chemical, St. Louis, MO, USA) and 10 mg/mL bovine serum albumin (BSA) (Sigma Chemical). BSA was added because most leukocytes bind strongly to protein-free filters and do not move even in the presence of powerful stimulants [27]. Chemotaxis was evaluated in a 12-well modified Boyden chamber (Neuroprobe) using a 150 μm thick cellulose nitrate membrane (pore size: 3 μm). The lower wells were filled with 150 μL of HBSS + BSA + HEPES (spontaneous movements), or with the following chemoattractants: Zymosan activated serum (ZAS), prepared by incubating (30 min at 37 °C) a pool of fresh ovine sera with 5 mg/mL of boiled Zymosan (Sigma Chemical) and diluted to 50% with HBSS + BSA + HEPES; 25 ng/mL or 50 ng/mL of human recombinant IL-8 77 amino acids (Peprotech Ltd, London, England) in HBSS + BSA + HEPES. After positioning the membrane, the upper wells were filled with 120 μL of the cell suspension. Each test was run in duplicate.

After incubation (1 h at 37 °C, 5% CO_2), the membrane was removed, fixed in ethanol 70°, hydrated in distilled water, stained in Mayer hematoxylin, dehydrated in ethanol (70°, 95° and absolute) and clarified in xylene. The parts of the membrane corresponding to the different wells were then excised and placed upside down on a microscope slide. Coverslips were mounted with a non-aqueous medium (Eukitt, Kindler, Freiburg, Germany).

Chemotaxis was evaluated at 400 × magnification, using the leading front method [28]: The distance from the top of the membrane (on which most of the PMN were in focus) to the furthest focal plane on which at least two cells were in focus (leading front) was measured with the optical micrometer of the fine focus knob of the microscope. Ten fields from each well were read independently by two observers. The results are the mean values of the 10 readings of each of the two observers for each of the duplicates incubated with the same chemoattractant.

2.5. Phagocytosis

Two different methods for the evaluation of phagocytosis were used:

Phagocytosis of non-opsonized latex particles. PMN were diluted at a final concentration of 10^7 cells/mL in 1 mL of HBSS and incubated (37 °C, 30 min) with polystyrene latex beads (Sigma Chemical) (diameter 1.09 μm, 20 particles per PMN in 1 mL of HBSS) [23]. Phagocytosis was stopped with 4 mL of PBS containing 40 mmol/L EDTA. Cells were then washed twice with PBS to eliminate the uningested particles. The ingested latex was solubilized with 2 mL of undiluted 1-4 dioxan (Sigma Chemical) (20 h at 23 °C). The amount of ingested latex was estimated in a spectrophotometer (Jasco V530) using a wavelength of 255.5 nm, against a blank containing only 1-4 dioxan and the values were expressed as optical densities (O.D.)

Phagocytosis of opsonized and fluoresceinated Saccaromyces cerevisiae *yeasts* [26]. Yeast particles (Sigma Chemical) were boiled in a water bath for 30 min, filtered, washed twice with PBS and diluted at 4×10^{11} cell/L. Particles were then marked with fluoresceine isothiocianate (FITC) (Sigma Chemical) (0.25 g FITC/L in 50 mmol/L carbonate buffer pH 10), washed twice in PBS, adjusted to 10^{13} cell/L and stored at –20 °C. Immediately before

each test session, 100 μL of yeast suspension were thawed, diluted at 5×10^{10} cell/L, opsonized by incubation for 30 min at 37 °C with the same volume of pooled ovine serum (50% in PBS), washed in PBS and resuspended in HBSS at 3×10^9 cell/L.

For particle uptake, 100 μL of PMN (6×10^4/mL) were incubated (30 min at 37 °C, in 5% CO_2 atmosphere) on slides on which grease-pencilled (Pap pen, Bio-optica, Milano, Italy) wells (average diameter = 1 cm) were drawn. The wells on the slides were then washed twice with PBS to remove non-adherent cells. After adding 100 μL of the yeast suspension to each well, the slides were incubated (30 min, 37 °C, 5% CO_2). The wells were then washed twice with cold PBS to stop phagocytosis and to remove non-ingested yeasts. Three drops of trypan blue solution were added to each well and discharged after a few seconds: trypan blue enters unphagocytized yeasts that lose their fluorescence, but it is expelled from live PMN so that phagocytized yeasts retain their fluorescence. The slides were then examined with a fluorescence microscope and the number of ingested particles per PMN was evaluated in 100 to 200 cells per slide. The phagocytic index (PI) was then calculated as the mean number of ingested yeast/PMN. The slides were then stained with May Grünwald-Giemsa and the percentage of cells in phagocytosis was counted under a light microscope as follows: (number of PMN with intra-cytoplasmic yeasts/total PMN number) × 100.

2.6. Adherence and superoxide production

These parameters were simultaneously evaluated in a 96 well microtiter plate as previously described [24]. Since the contact with plastic wells could non-specifically activate PMN adherence and O_2^- production, one half of the wells were gelatin-coated, by incubation (60 min, 4 °C) with gelatin (Sigma Chemical) (5mg/mL PBS) followed by two washes with PBS [15]. The other wells were used to evaluate non-specific activation by plastic contact. 100 μL of cell suspension (final dilution: 2×10^5/mL) were put in each well and warmed for 10 min at 37 °C in humidified CO_2 atmosphere. 50 μL of cytochrome C (Sigma Chemical) (0.054 mmol/L in HBSS) were then added to each well. In one half of the gelatin-coated and in one half of the gelatin free wells phorbol myristate acetate (PMA) (Sigma Chemical) (10^{-7} mol/L) was added. Each test was carried out in quadruplicate. Thus, four groups of samples were obtained:

gelatin coated wells without PMA: basal activity;

gelatin coated wells with PMA: specific activation by PMA;

wells without gelatin and without PMA: non-specific activation by plastic;

wells without gelatin and with PMA: simultaneous activation by plastic and PMA.

O_2^- production was measured by superoxide dismutase (SOD) inhibitable reduction of ferricytochrome C: to verify the specificity of superoxide production, a blank well containing 700 U of SOD (Sigma Chemical) other than the previously mentioned compounds was prepared.

Superoxide production was evaluated by measuring the optical density using an automatic ELISA analyzer (Dasit Multiskan MS, Dasit, Cornaredo, MI, Italy) at 550 nm. As the wavelength reference, the plates were also read at 670 nm. Readings were carried out at the beginning of the test, then the plates were incubated (37 °C in 5% CO_2) and the readings were repeated after 40 min. The nanomoles of SO_2^- produced were then calculated as follows: [(OD550-OD670) × 100]/6.3.

After 60 min of incubation, adherence was evaluated: the plates were washed twice with PBS to remove non-adherent cells. In each well 75 μL of acetate buffer 0.15 M,

pH 5.3, containing 0.2% of Triton X-100 (Sigma Chemical) were added. After 5 min of incubation at room temperature to allow the lysis of adherent cells, 75 µl of acetate buffer 0.1 M, pH 5.3, containing 10 mmol/L of p-nitrophenilphosphate (Sigma Chemical), the substrate for acid phosphatase, were added. After 20 min of incubation at room temperature the reaction was stopped by adding 100 µL of NaOH 2N and optical density was determined against blank at 405 nm in an automatic ELISA analyzer (Dasit Multiskan MS).

2.7. Statistical analysis

Statistical analysis was performed using a specific software (Statsoft inc., Tulsa, OK, USA). The results obtained for both groups were compared to each other with unpaired Student t-test or, when normality tests showed that the data did not have normal distribution, with the corresponding U-Mann Whitney test. Data recorded at the different time intervals in each group were compared by ANOVA for repeated measurements or with the corresponding non-parametric Friedman test, followed by the Tukey Honest Significant Difference (HSD) test. The same tests were used to evaluate the effects of the different activators on chemotaxis, adherence and O_2^- production.

The correlations between percentage and number of leukocytes in blood and in the isolated cell population were investigated using the Spearman correlation test. The same test was used to correlate the percentage of cells in the isolated cell population and the results of functional tests on PMN. Only correlations with r close to or higher than 0.5 were considered as biologically significant.

In all the above mentioned tests a P value < 0.05 was considered significant.

3. RESULTS

3.1. Clinical chemistry and hematology (Tabs. I and II)

Clinical, biochemical and hematological parameters from control sheep did not show any significant difference among the five samplings, except for a decrease of NEFA at T24 and T48.

The administration of 6.5 µg/kg b.w. of 1-24ACTH induced a significant increase of clinical parameters and of cortisol at T1 and T2. Glucose and NEFA increased at T2. Furthermore, treated animals had increased leukocytes and neutrophils at T2, when the PMN number was higher than in the controls.

3.2. PMN isolation (Tab. III)

In the controls, no differences in the mean values of the number of isolated cells/µL of cell suspension were detectable among the different samplings. PMN were the most represented cell type but eosinophils and lymphocytes were also present.

The number of leukocytes and the percentage of PMN isolated from treated sheep increased at T2, when they were also significantly higher than the value recorded in the controls. PMN viability and recovery rate did not change in the five time intervals, nor were they different between the two groups. The recovery rate was negatively correlated with the number of circulating PMN (r = –0.42).

3.3. Chemotaxis (Tab. IV)

In both controls and treated sheep, the distance moved in the presence of the different chemokines was always significantly higher than in their absence. The distance moved against IL-8 at T2 was significantly higher than at T0 in the controls but not in the treated sheep. No correlations were

Table I. Clinical and biochemical parameters (means ± S.D., $n = 10$) recorded in controls (C) and in 1-24ACTH treated sheep (T).

		T0	T1	T2	T24	T48	ANOVA	Tukey vs. T0
temperature (°C)	C	38.6 ±0.3	38.7 ± 0.2	38.7 ± 0.4	38.6 ± 0.3	38.7 ± 0.2	ns	ns
	T	38.7 ±0.2	39.0 ± 0.3	39.2 ± 0.2	38.7 ± 0.2	38.9 ± 0.1	*	T1, T2
		ns	*	*	ns	ns		
Heart rate (beats/min)	C	52.5 ±4.1	52.6 ± 3.8	56.9 ± 6.4	53.1 ± 5.5	59.8 ± 7.2	ns	ns
	T	52.6 ±3.7	60.4 ± 4.8	69.2 ± 4.7	77.2 ± 12.7	68.8 ± 9.9	*	T2, T24, T48
		ns	*	*	*	*		
Cortisol (ng/mL)	C	6.2 ±3.0	5.1 ± 1.9	5.5 ± 2.6	6.0 ± 2.2	6.0 ± 2.2	ns	ns
	T	6.8 ± 4.0	58.7 ± 19.9	46.8 ± 23.2	14.5 ± 21.9	9.1 ± 9.1	*	T1, T2
		ns	*	*	ns	ns		
NEFA (mEq/L)	C	83.7 ± 31.2	84.0 ± 26.0	69.7 ± 25.8	65.2 ± 27.1	65.4 ± 16.5	*	T24, T48
	T	63.2 ± 28.5	83.2 ± 45.4	112.5 ± 65.1	120.3 ± 73.5	58.5 ± 21.8	*	T2, T24
		ns	ns	ns	ns	ns		
Glucose (mg/dL)	C	57.6 ± 6.7	59.4 ± 7.4	64.5 ± 10.9	62.5 ± 6.3	58.7 ± 5.6	ns	ns
	T	59.7 ± 5.0	62.5 ± 8.2	65.8 ± 9.8	62.1 ± 6.7	58.6 ± 4.2	*	T2
		ns	ns	ns	ns	ns		

* $P < 0.05$; ns: not significant.

Table II. Hematological parameters (means ± S.D., $n = 10$) recorded in controls (C) and in 1-24ACTH treated sheep (T).

		T0	T1	T2	T24	T48	ANOVA	Tukey vs. T0
Leukocytes × $10^3/\mu L$	C	7.8 ± 2.5	7.6 ± 2.3	7.7 ± 2.8	6.9 ± 2.5	7.2 ± 2.8	ns	ns
	T	8.6 ± 2.7	8.3 ± 2.3	9.5 ± 2.4	7.9 ± 2.3	7.9 ± 2.2	*	T2
		ns	ns	ns	ns	ns		
Neutrophils × $10^3/\mu L$	C	2.3 ± 1.2	2.0 ± 0.6	2.5 ± 1.2	2.1 ± 0.9	2.1 ± 1.0	ns	ns
	T	3.3 ± 2.2	3.0 ± 1.6	4.9 ± 1.7	2.5 ± 1.3	2.4 ± 1.0	*	T2
		ns	ns	*	ns	ns		
Eosinophils × $10^3/\mu L$	C	0.7 ± 0.7	0.6 ± 0.6	0.8 ± 0.8	0.6 ± 0.6	0.5 ± 0.5	ns	ns
	T	0.6 ± 0.3	0.5 ± 0.4	0.4 ± 0.3	0.5 ± 0.3	0.6 ± 0.4	ns	ns
		ns	ns	ns	ns	ns		
Lymphocytes × $10^3/\mu L$	C	4.5 ± 1.0	4.9 ± 1.7	4.2 ± 1.0	4.0 ± 1.3	4.4 ± 1.5	ns	ns
	T	4.6 ± 1.2	4.6 ± 1.2	4.1 ± 1.1	4.6 ± 1.5	4.7 ± 1.3	ns	ns
		ns	ns	ns	ns	ns		
Monocytes × $10^3/\mu L$	C	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	ns	ns
	T	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	ns	ns
		ns	ns	ns	ns	ns		

* $P < 0.05$; ns: not significant.

Table III. Characteristic of the isolated cell populations (means ± S.D., $n = 10$) recorded in controls (C) and in 1-24ACTH treated sheep (T).

		T0	T1	T2	T24	T48	ANOVA	Tukey vs. T0
Isolated cells × 10^3/μL	C	45.0 ± 21.5	44.7 ± 20.1	47.5 ± 20.3	42.4 ± 23.0	45.5 ± 21.4	ns	ns
	T	47.4 ± 22.2	56.1 ± 23.0	75.6 ± 19.8	46.7 ± 21.1	47.7 ± 24.1	*	T2
		ns	ns	*	ns	ns		
Neutrophils (%)	C	71.3 ± 17.0	69.9 ± 13.3	72.1 ± 12.8	66.6 ± 16.9	68.4 ± 11.2	ns	ns
	T	70.9 ± 15.4	73.5 ± 10.3	86.1 ± 10.7	76.5 ± 12.8	74.0 ± 15.0	*	T2
		ns	ns	*	ns	ns		
Eosinophils (%)	C	18.6 ± 16.8	18.2 ± 16.0	15.9 ± 13.0	18.8 ± 17.6	17.1 ± 11.8	ns	ns
	T	15.8 ± 10.9	15.3 ± 7.8	7.3 ± 5.4	14.4 ± 11.0	11.9 ± 9.3	*	T2
		ns	ns	*	ns	ns		
Lymphocytes (%)	C	9.9 ± 7.9	10.9 ± 6.2	10.7 ± 6.4	14.2 ± 12.1	13.3 ± 4.7	ns	ns
	T	12.4 ± 9.1	10.8 ± 5.3	6.0 ± 6.3	7.4 ± 5.3	12.1 ± 11.8	ns	ns
		ns	ns	ns	ns	ns		
Monocytes (%)	C	0.2 ± 0.4	1.0 ± 1.6	0.3 ± 0.5	0.4 ± 0.5	1.0 ± 1.2	ns	ns
	T	0.9 ± 1.0	0.4 ± 0.5	0.5 ± 0.7	1.7 ± 3.1	2.0 ± 2.6	ns	ns
		ns	ns	ns	ns	ns		
PMN recovery rate (%)	C	41.0 ± 23.2	39.1 ± 16.5	36.1 ± 14.2	33.9 ± 13.0	38.6 ± 14.2	ns	ns
	T	31.6 ± 16.1	37.9 ± 13.5	35.2 ± 10.4	36.9 ± 7.7	36.5 ± 13.9	ns	ns
		ns	ns	ns	ns	ns		
PMN viability (%)	C	97.3 ± 1.6	97.0 ± 2.0	97.1 ± 1.2	97.0 ± 1.7	97.1 ± 2.0	ns	ns
	T	97.2 ± 0.6	97.2 ± 1.1	97.1 ± 0.3	97.3 ± 0.7	97.2 ± 0.6	ns	ns
		ns	ns	ns	ns	ns		

* $P < 0.05$; ns: not significant.

Table IV. Results (means ± S.D.) of chemotaxis tests recorded in controls (C) and in 1-24ACTH treated sheep (T).

		T0	T1	T2	T24	T48	ANOVA	Tukey vs. T0
Basal (μm)	C	52.4 ± 7.0	56.1 ± 6.3	58.4 ± 4.3	51.3 ± 8.2	54.8 ± 9.0	ns	ns
	T	51.6 ± 8.8	52.7 ± 13.5	53.5 ± 13.7	53.9 ± 14.5	50.8 ± 12.9	ns	ns
		ns	ns	ns	ns	ns		
ZAS (μm)	C	59.5 ± 5.8 §	62.1 ± 5.4 §	66.8 ± 5.7 §	58.8 ± 9.3 §	61.3 ± 6.6 §	ns	ns
	T	58.8 ± 8.8 §	62.1 ± 9.9 §	64.8 ± 9.6 §	62.9 ± 8.7 §	66.2 ± 10.3 §	ns	ns
		ns	ns	ns	ns	ns		
IL8 25 ng/mL (μm)	C	59.6 ± 8.6 §	65.0 ± 6.6 §	68.6 ± 5.7 §	59.7 ± 8.9 §	61.4 ± 8.8 §	*	T2
	T	56.7 ± 12.2 §	60.7 ± 15.3 §	62.7 ± 14.9 §	61.6 ± 14.2 §	63.2 ± 14.3 §	ns	ns
		ns	ns	ns	ns	ns		
IL8 50 ng/mL (μm)	C	62.6 ± 6.8 §	66.8 ± 7.1 §	70.9 ± 5.3 §	59.6 ± 8.9 §	62.6 ± 9.0 §	*	T2
	T	59.4 ± 11.6 §	60.0 ± 17.0 §	64.7 ± 16.7 §	61.3 ± 14.6 §	63.2 ± 15.2 §	ns	ns
		ns	ns	ns	ns	ns		

Basal: spontaneous movements; ZAS: activation with Zymosan activated serum; IL-8 25 ng/mL: activation with 25 ng/mL of interleukin-8; IL-8 50 ng/mL: activation with 50 ng/mL of interleukin-8.
* $P < 0.05$; § $P < 0.05$ vs. basal chemotaxis in the same group; ns: not significant.

found between chemotaxis and the percentage of PMN in the isolated cell population.

3.4. Phagocytosis (Tab. V)

The amount of ingested latex in controls at T48 was significantly lower compared to T1 and T2, but no differences between the two groups of sheep were detectable. The percentage of PMN phagocytosing opsonized and fluoresceinated yeasts and the phagocytic index (PI) were not significantly different among the five time samplings nor between the two groups of animals. A positive correlation ($r = 0.91$) between PI and the percentage of cells in phagocytosis was detectable.

3.5. Adherence (Tab. VI)

This parameter showed a strong individual variability. PMA induced a significant increase of adherence in controls and at T2 the non-specific stimulation also increased the adhesive properties of ovine PMN. No differences among the five samplings were detectable in any of the experimental conditions.

In 1-24 ACTH treated sheep, the adhesive response to PMA significantly increased at T2, when the values recorded in the presence of PMA were also significantly higher than those recorded with non-specific activation. Furthermore, the non-specific activation induced an increase of adherence at T24. No correlations between the percentage of isolated PMN and the results of adherence tests were detectable.

3.6. Superoxide production (Tab. VII)

In controls, the unstimulated O_2^- production significantly increased at T48 and the PMA-activated O_2^- production at T24 and T48. PMA strongly increased O_2^- production in both groups. No significant differences between the five time samplings were detectable in the treated sheep, nor between the control and treated sheep.

4. DISCUSSION

Control sheep did not show any biochemical or hematological changes compared to those of the literature [9, 11], except for a moderate decrease of NEFA at T24 and T48. The administration of 6.5 μg/kg b.w. 1-24ACTH induced a strong acute stress reaction, as indicated by the clinical, biochemical and hematological data, which were in compliance with previous reports in sheep [9, 14].

Table V. Results (means ± S.D.) of phagocytosis tests recorded in controls (C) and in 1-24ACTH treated sheep (T).

		T0	T1	T2	T24	T48	ANOVA	Tukey vs. T0
Phagocytic index (PI)	C	0.93 ± 0.53	0.94 ± 0.62	0.92 ± 0.58	0.80 ± 0.37	0.96 ± 0.63	ns	ns
	T	1.16 ± 0.70	1.09 ± 0.60	1.33 ± 0.76	1.14 ± 0.67	1.27 ± 0.47	ns	ns
		ns	ns	ns	ns	ns		
Phagocytosing PMNs (%)	C	57.7 ± 18.8	53.9 ± 23.2	54.3 ± 23.9	52.4 ± 16.9	56.6 ± 25.1	ns	ns
	T	28.8 ± 25.9	60.9 ± 22.1	66.8 ± 23.8	61.3 ± 24.4	63.2 ± 14.0	ns	ns
		ns	ns	ns	ns	ns		
Ingested latex (O.D.)	C	0.52 ± 0.20	0.62 ± 0.26	0.62 ± 0.29	0.38 ± 0.24	0.31 ± 0.19	*	ns
	T	0.55 ± 0.26	0.59 ± 0.31	0.72 ± 0.42	0.54 ± 0.17	0.51 ± 0.30	ns	ns
		ns	ns	ns	ns	ns		

O.D.: optical density; * $P < 0.05$; ns: not significant.

Table VI. Results (means ± S.D.) of adherence tests recorded in controls (C) and in 1-24ACTH treated sheep (T).

		T0	T1	T2	T24	T48	ANOVA	Tukey vs. T0
Unstimulated (O.D.)	C	0.13 ± 0.20	0.20 ± 0.28	0.11 ± 0.11	0.15 ± 0.16	0.14 ± 0.12	ns	ns
	T	0.09 ± 0.11	0.08 ± 0.09	0.13 ± 0.22	0.14 ± 0.09	0.15 ± 0.18	ns	ns
		ns	ns	ns	ns	ns		
Non specific (O.D.)	C	0.25 ± 0.29	0.30 ± 0.28	0.25 ± 0.13§	0.25 ± 0.20	0.28 ± 0.21	ns	ns
	T	0.15 ± 0.15	0.20 ± 0.18	0.23 ± 0.24	0.25 ± 0.20§	0.23 ±.0.20	ns	ns
		ns	ns	ns	ns	ns		
PMA (O.D.)	C	0.31 ± 0.37§	0.39 ± 0.37§	0.41 ± 0.26§	0.27 ± 0.18§	0.34 ± 0.33§	ns	ns
	T	0.23 ± 0.17§	0.26 ± 0.19§	0.42 ± 0.20§#	0.32 ± 0.16§	0.26 ± 0.17§	*	T2
		ns	ns	ns	ns	ns		
Mixed (O.D.)	C	0.35 ± 0.39§	0.39 ± 0.33§	0.44 ± 0.26§	0.28 ± 0.18§	0.36 ± 0.31§	ns	ns
	T	0.24 ± 0.18§	0.29 ± 0.21§	0.43 ± 0.19§#	0.36 ± 0.19§	0.27 ± 0.17§	*	T2
		ns	ns	ns	ns	ns		

Unstimulated: no activation; Non specific: activation by plastic contact; PMA: activation by phorbolmyristate acetate (PMA); Mixed: activation by both plastic contact and PMA.
O.D.: Optical density; * $P < 0.05$; § $P < 0.05$ vs. unstimulated adherence in the same group; # $P < 0.05$ vs. non specific activation in the same group; ns: not significant.

Table VII. Results (means ± S.D.) of O_2^- production tests recorded in controls (C) and in 1-24ACTH treated sheep (T).

		T0	T1	T2	T24	T48	ANOVA	Tukey vs. T0
Unstimulated (nanomoles)	C	0.15 ± 0.11	0.12 ± 0.13	0.14 ± 0.17	0.30 ± 0.17	0.37 ± 0.28	*	T48
	T	0.19 ± 0.19	0.13 ± 0.12	0.16 ± 0.07	0.13 ± 0.17	0.25 ± 0.12	ns	ns
		ns	ns	ns	ns	ns		
Non specific (nanomoles)	C	0.34 ± 0.17	0.30 ± 0.25	0.46 ± 0.41	0.50 ± 0.33	0.57 ± 0.31	ns	ns
	T	0.37 ± 0.28	0.30 ± 0.23	0.34 ± 0.23	0.37 ± 0.33	0.43 ± 0.20	ns	ns
		ns	ns	ns	ns	ns		
PMA (nanomoles)	C	1.83 ± 0.54§#	1.88 ± 0.62§#	2.37 ± 0.67§#	2.56 ± 1.09§#	2.94 ± 0.96§#	*	T24, T48
	T	2.52 ± 0.91§#	2.23 ± 1.01§#	2.76 ± 0.73§#	3.01 ± 0.77§#	2.85 ± 0.39§#	ns	ns
		ns	ns	ns	ns	ns		
Mixed (nanomoles)	C	2.23 ± 0.84§#	1.75 ± 0.56§#	1.89 ± 0.56§#	2.63 ± 1.08§#	2.82 ± 0.80§#	*	ns
	T	2.88 ± 1.03§#	2.06 ± 0.92§#	2.13 ± 0.55§#	2.98 ± 0.86§#	2.64 ± 0.50§#	ns	ns
		ns	ns	ns	ns	ns		

Unstimulated: no activation; Non specific: activation by plastic contact; PMA: activation by both plastic contact and phorbolmyristate acetate (PMA); Mixed: activation by both plastic contact and PMA;
* $P < 0.05$; § $P < 0.05$ vs. unstimulated O_2^- production in the same group; # $P < 0.05$ vs. non specifically activated O_2^- production in the same group; ns: not significant.

The isolation technique allowed us to obtain a good cell population in terms of cell viability, PMN purity and recovery rate. However, as previously reported [23], PMN were not selectively isolated, and lymphocytes and eosinophils were always present in the isolated cell populations, yet, both these populations do not have phagocytic ability nor do they respond to the chemokines used here without a previous cytokine priming [16]. In contrast, they could have interfered with adherence and O_2^- production evaluation [4, 5]. The negative correlation between the recovery rate and the number of circulating leukocytes might depend on the presence of circulating

PMN with low density: segmented neutrophils released after 1-24 ACTH administration might be less mature than normal and, according to Glasser and Fiederlein [6], hypodense.

As previously reported [23], both ZAS and IL-8 are good activators of ovine PMN movements. The responsiveness to these compounds increased in controls from T0 to T2, probably due to receptorial or post-receptorial changes in PMN [20]. Administration of 1-24ACTH caused the lack of these differences, suggesting that during stress, neutrophils are less reactive to chemokines. These results are in compliance with those detected in sheep during chronic stress but not with other studies, most likely depending on different experimental protocols [10, 21].

The uptake of latex beads showed only minor changes in controls and none in treated sheep. However, the lack of differences in the ingestion of opsonized particles, more representative of the in vivo process than the ingestion of non- opsonized particles, suggests that phagocytosis is not influenced in vivo.

The lack of significant differences among the different samplings in adherence tests from control sheep might be due to the high variability recorded. PMA is a good activator of adherence in ovine PMN, as already reported [24]. Adherence increased 2 h after 1-24ACTH administration. The lack of correlation with the percentage of PMN in the isolated cell population allows to exclude that such changes depend on the presence of a higher percentage of isolated PMN. These results seem to be in contrast with the finding of a decrease of L-selectin and of CD18 in dexamethazone-treated calves [12] and with the well known mechanism of stress-induced neutrophilia [9]: during stress, in fact, the adherence between neutrophils of the marginal pool and endothelial cells decreases. However this might depend on changes in the expression of endothelial mediators or adhesion molecules in spite of an increase in the adhesive properties of PMN [1].

The O_2^- production at T0 in controls was quantitatively similar to that recorded in a previous work in which a high individual variability, and the decrease of this variability after activation, were also reported [24]. Such findings are confirmed by the present work. The increase of O_2^- production in controls at T48 can be due to the presence of PMN released from bone marrow as a consequence of repeated samplings: early segmented PMN with different functional activities compared with completely mature ones have been in fact previously reported [6]. The lack of this increase in treated sheep seems to support a previously reported stress-induced depression of O_2^- production [8]. Administration of 1-24ACTH, in contrast, did not influence the oxydative responses of PMN to the different activators.

In conclusion, the dose of 1-24 ACTH employed in this study induced clinical, biochemical and hematological changes consistent with a stress reaction. Among the different functional activities of PMN, only adherence increased 24 to 48 h after 1-24ACTH administration. This might depend on the effect of ACTH and cortisol or on the release from the bone marrow or from the marginal pool of PMN with altered functions. Further elucidations might be acquired using stressors other than the simple 1-24ACTH administration, or by studies that can take the influence of lymphocytes or endothelial cells into account.

ACKNOWLEDGEMENTS

The authors thank Dr. Roberta Nicoli and Mrs. Sonia Dominoni for their technical assistance.

REFERENCES

[1] Arber N., Berliner S., Rotenberg Z., Friedman J., Belagodatni E., Ostfeld I., Aronson M., Pinkhas J., Detection of aggregated leukocytes in the circulating pool during stress-demargination is not necessary a result of decreased leukocyte adhesiveness, Acta Haematol. 86 (1991) 20-24.

[2] Buchta R., Functional and biochemical properties of ovine neutrophils, Vet. Immunol. Immunopathol. 24 (1990) 97-112.

[3] Carlson G.P., Kaneko J.J., Isolation of leukocytes from bovine peripheral blood, Proc. Soc. Exp. Biol. Med. 142 (1973) 853-856.

[4] Foster A.P., Cunningham F.M., Differential superoxide anion generation by equine eosinophils and neutrophils, Vet. Immunol. Immunopathol. 59 (1997) 225-237.

[5] Garrido E., Delgado Martinez C., Gomariz R.P., De La Fuente M., Pituitary adenylate cyclase-activating polypeptide (PACAP38) modulates lymphocyte and macrophage functions: stimulation of adherence and opposite effect on mobility, Neuropeptides 30 (1996) 583-595.

[6] Glasser L., Fiederlein R.L., Functional differentiation of normal human neutrophils, Blood 69 (1987) 937-944.

[7] Gwazdauskas F.C., Paape M.J., Perry D.A., McGilliard M.L., Plasma glucocorticoid and circulating blood leukocyte responses in cattle after sequential intramuscular injections of ACTH, Am. J. Vet. Res. 41 (1980) 1052-1056.

[8] Henricks P.A.J., Binkhorst G.J., Nijkamp F.P., Stress diminishes infiltration and oxygen metabolism of phagocytic cells in calves, Inflammation 11 (1987) 427-437.

[9] Jain N.C., Schalm's Veterinary Hematology, 4th ed., Philadelphia Lea and Febiger, 1986.

[10] Jayappa H.G., Loken K.I., Effect of antimicrobial agents and corticosteroids on bovine polymorphonuclear leukocyte chemotaxis, Am. J. Vet. Res. 44 (1983) 2155-2159.

[11] Kaneko J.J., Harvey J.W., Bruss M.L., Blood analyte reference values in small and some laboratory animals, in: Kaneko J.J., Harvey J.W., Bruss M.L. (Eds.), Clinical biochemistry of domestic animals, 5th ed., Academic Press, San Diego, 1997, pp. 895-899.

[12] Kehrli M.E., Burton J.L., Nonnecke B.J., Lee E.K., Effects of stress on leukocyte trafficking and immune responses: implications for vaccination, Adv. Vet. Med. 41 (1999) 61-81.

[13] Kelley KW., Stress and immune function: a bibliographic review, Ann. Rech. Vet. 11 (1980) 445-478.

[14] Locatelli A., Agnes F., Sartorelli P., Tozzi F., Arrigoni C., Rilievi ematochimici in ovini sottoposti a inoculazione di 1-24 ACTH e a trasporto simulato, Clin. Vet. 108 (1985) 345-351.

[15] Metcalf J.A., Gallin J.I., Nauseef W.M., Root R.K., Laboratory manual of neutrophil function, Raven Press, New York, 1986.

[16] Mukaida N., Harada A., Matsushima K., Interleukin-8 (IL-8) and Monocyte Chemotactic and Activating Factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions, Cytokine Growth Factor Rev. 9 (1998) 9-23.

[17] Murata H., Hirose H., Suppression of bovine neutrophil function by sera from cortisol-treated calves, Br. Vet. J. 147 (1991) 63-70.

[18] Ndibualonji B.B., Dehareng D., Van Eenaeme G., Godeau J.M., Response of milk yield, plasma cortisol, amino acids, urea and glucose to a single low-dose administration of adrenocorticotrophic hormone in lactating cows, Vet. Res. 26 (1995) 32-42.

[19] Paape M.J., Gwazdauskas F.C., Guidry A.J., Weinbland B.T., Concentrations of corticosteroids, leukocytes, and immunoglobulins in blood and milk after administration of ACTH to lactating dairy cattle: effects on phagocytosis of *Staphilococcus aureus* by polymorphonuclear leukocytes, Am. J. Vet. Res. 42 (1981) 2081-2087.

[20] Paltrinieri S., Panelli S., Sartorelli P., Characteristics of the response of ovine granulocytes (PMNs) to zymosan activated serum (ZAS) and to recombinant human interleukin-8 (IL-8), J. Vet. Med. A 47 (2000) 421-430.

[21] Roth J.A., Kaeberle M.L., Hsu W.H., Effects of ACTH administration on bovine polymorphonuclear leukocyte function and lymphocyte blastogenesis, Am. J. Vet. Res. 43 (1982) 412-416.

[22] Sartorelli P., Arrigoni C., Paltrinieri S., Ruggiero M.R., Agnes F., Zinco, rame e ceruloplasmina nel vitello e nella pecora dopo stimolazione corticosurrenalica, Arch. Vet. Ital. 44 (1993) 135-140.

[23] Sartorelli P., Paltrinieri S., Agnes F., Non specific Immunity and ketone bodies. I. "In vitro" studies on chemotaxis and phagocytosis in ovine neutrophils, J. Vet. Med. A 46 (1999) 613-619.

[24] Sartorelli P., Paltrinieri S., Comazzi S., Non specific Immunity and ketone bodies. II. "In vitro" studies on adherence and superoxide anion production in ovine neutrophils, J. Vet. Med. A 47 (2000) 1-8.

[25] Slein M.W., D glucose: determination with hexokinase and glucose-6-phospate dehydrogenase, in: Bergmeyer H.U. (Ed.), Methods of enzymatic analysis, 2nd ed., Verlag Chemie, Weinheim, Academic Press, London, 1965, pp. 117-123.

[26] Trowald-Wight G., Thoren-Tholling K., Phagocytosis induced by yeast cells in canine granulocytes: a methodological study, Acta Vet. Scand. 31 (1990) 87-95.

[27] Wilkinson P.C., Micropore filter methods for leukocyte chemotaxis, Methods Enzymol. 162 (1988) 38-50.

[28] Zigmond S.H., Hirsch J.G., Leukocyte locomotion and chemotaxis, J. Exp. Med. 137 (1973) 387-410.