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Original Article

In vitro assessment of the effects of temperature on phagocytosis, reactive oxygen species production and apoptosis in bovine polymorphonuclear cells

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Abstract

Heat stress exerts a direct negative effect on farm animal health, triggering physiological responses. Environmental high temperature induces immunosuppression in dairy cows, increasing the risk of mastitis and milk somatic cell counts. The influence of heat stress on leukocytes activities have not been fully elucidated. The present *in vitro* study was aimed at assessing whether the exposure to temperature simulating conditions of severe whole body hyperthermia affects defensive functions of bovine blood polymorphonuclear cells.

Blood was collected from seven clinically healthy, multiparous, late lactating Holstein cows. After isolation, PMN were incubated at either 39 or 41 °C. Phagocytosis, respiratory burst and apoptosis were then investigated. The selected temperatures of 39 °C or 41 °C mimicked conditions of normothermia or severe heat stress, respectively.. Phagocytosis assay was carried out by measuring the fluorescence of phagocyted fluorescein-labelled *E. coli* bioparticles. The modulation of oxidative burst activity was studied by the cytochrome C reduction method. Apoptosis was determined by measuring the activities of two enzymes that play an effector role in the process, namely Caspase-3 and Caspase-7. Statistical analyses were performed using SPSS 22.0. A Student t-test for paired samples and a Generalised Estimating Equation were used based on data distribution.

, The phagocytosis rate was reduced (-37%, $P<0.01$) when PMN were incubated for 2 h at 41°C, when compared to phagocytosis rate measured at 39 °C. The oxidative burst, as determined by extracellular production of reactive oxygen species (ROS), was also reduced by the exposure of cells to 41°C compared to 39 °C. Such reduction ranged between -2 and -21% ($P<0.05$). Apoptosis rate was not affected by different temperatures.

41 The results reported in this study suggest that phagocytosis and ROS production in PMN
42 exposed to severe high temperature are impaired, partially explaining the higher occurrence of
43 infections during periods of hot weather.

44

45 *Key words*: bovine; polymorphonuclear cells; high temperature; apoptosis; ROS

46

1. Introduction

Heat stress (HS) induced immunosuppression is indicated as one of the mechanisms through which climate changes, more specifically the increase of air temperature, are expected to exert a direct negative effect on human and animal health in the next decades (Gaughan et al., 2009; Martin et al., 2010; Dang et al., 2012).

Studies focusing on the relationships between environmental temperature and health problems in dairy cows pointed out higher risks of mastitis (Giesecke 1985; Smith et al. 1985; Waage et al. 1998; Olde Riekerink et al., 2007) and an increase of milk somatic cell counts (Bertocchi et al., 2014) during periods of hot weather. The mechanisms behind the higher risk of mastitis during periods of HS have not been elucidated although it a negative action of HS on defensive mechanisms has been suggested as one of the most probable mechanism (Giesecke 1985; Bertocchi et al., 2014).

The effect of HS on cattle immune functions (enhancement, suppression, or no effect) depends on several factors, which include breed, genotype, age, acclimation level, intensity and duration of the exposure to high temperatures, recovery opportunities, the specific immune parameter taken into account, the experimental models adopted (*in vivo*, *ex vivo*, and *in vitro*) and their interactions (Lacetera, 2012). Severe HS has been shown to impair immune functions in dairy cows. In particular, both *ex vivo* and *in vitro* studies focusing on proliferation of mitogens stimulated peripheral blood mononuclear cells (PBMC) pointed out that high temperatures alter the ability of PBMC to proliferate (Lacetera et al., 2005, 2009). Furthermore, preliminary results from ongoing *in vitro* studies indicate that incubation temperatures simulating whole body severe hyperthermia (41 °C and above) impair the secretion of TNF-alpha and IFN-gamma from PBMC isolated from dairy cows (Lacetera et al., unpublished).

70 The effects of high temperatures on functions of bovine polymorphonuclear cells (PMN)
71 received only limited attention, despite their involvement in the protection against mastitis and
72 metritis (Mehrzad et al., 2010; LeBlanc et al., 2011). Elvinger and co-workers (1991) reported that
73 incubation temperature of 42 °C inhibited certain response of bovine PMN even if results from
74 different experiments were not clear and conclusive. Do Amaral and co-workers (2011) reported
75 that summer cooling may exert a positive effect on phagocytosis and oxidative burst of PMN
76 isolated from heat stressed dairy cows.

77 The apoptosis of PMN plays important roles in promoting resolution of the acute
78 inflammatory response (Headland and Norling, 2015). The impact of HS on PMN apoptosis has
79 been investigated in humans (Callahan et al., 1999; Kettritz et al., 2006; Nagarsekar et al., 2008;
80 Bzowska et al., 2011; Boyko et al., 2014). On the contrary, no information is available in bovine
81 species. Studies referred to humans focused on several aspects of PMN functions, which included
82 oxidants' production, heat shock response and apoptosis.

83 The present *in vitro* study was aimed at assessing whether exposure to temperature
84 simulating conditions of severe whole body hyperthermia affects functions of PMN in dairy cattle.

85

86 **2. Materials and Methods**

87 2.1 *Materials*

88 HEPES, RPMI-1640, HBSS with Mg^{++} and Ca^{++}, Red Blood Cell Lysing Buffer Hybri-Max,
89 PMA (phorbol 12-myristate 13-acetate), Cytochrome *c*, Percoll, trypan blue, PBS without Ca^{++} and
90 Mg^{++} and endotoxin-free water were purchased from Sigma-Aldrich Co. (St.Louis, MO, USA).
91 Fetal Bovine Serum was provided by Biochrom AG (Berlin, Germany) and Apo-ONE® Homogeneous Caspase-3/7 Assay from Promega Corporation (Milano, Italy). Ninety-six wells
92 sterile cell culture plates and 384 wells black sterile cell culture plates were purchased from Nunc
93

(Rochester, NY, USA). *Escherichia coli* (K-12 strain) BioParticles®, fluorescein conjugate, was provided by Molecular Probes (Invitrogen, San Giuliano Milanese (Milano), Italy).

2.2 *Source and PMN isolation*

The experiment was carried out on cells which were used for *in vitro* studies. Blood was collected from seven clinically healthy multiparous (2nd and 3rd calving), pregnant and late lactating (265 ± 50 days in milk) Holstein Friesian cows. Animals used in this study were managed according to the National Law for Animal Welfare and Protection (Italy). Late lactating cows were selected in order to avoid conditions of negative energy balance, which are known to interfere with immune functions. Cows used in the study did not suffer from clinical diseases in the last month before bleeding and somatic cell count at the time of blood collection was lower than 200,000 cells/ml. The experiment was carried out in March when cows were not exposed to heat stress conditions. In detail, during the sampling period average daily temperature was 8 ± 1.2 °C with 0 and 15 °C as minimum and maximum temperatures, respectively. Cows were housed and fed in free stalls, had free access to water, and were milked twice daily at 0600 and 1800 h.

Blood was collected by jugular venipuncture into sterile tubes containing 3.2% sodium citrate as anticoagulant and was processed within 1h of collection. Bovine PMN were isolated as described by Smits and coworkers (2000) with only minor modifications. Samples containing less than 5% of eosinophils were used. Blood was centrifuged at 1000 x g for 30 min at 4°C: The plasma, buffy coat and top one-third of the red blood cell pellet were removed. The remainder of pellet containing PMN was diluted 1:3 vol:vol in sterile cold PBS, gently layered on 10 ml Percoll 1.087 g/ml and centrifuged at 400 x g for 40 min at room temperature. After the removal of Percoll, the remaining red blood cells were removed by hypotonic lysis (Pisani et al., 2009) and PMN were washed four times with sterile cold PBS. All functional assays were performed simultaneously on the same day for any cow included in the present study.

2.3 *Phagocytosis assay*

Opsonisation of fluorescein-labelled *Escherichia coli* (K-12 strain) bioparticles was carried out by incubating the bacterial suspension ($6x10^8$) with 20% autologous serum for 30 min on a rocking roller at 37°C. The suspension was centrifuged at 800 x g for 15 min and suspended in sterile HBSS. Opsonised *E. coli* were stored at -20°C until use.

The concentration of PMN was adjusted to $3x10^6$/ml and 100µl of this solution ($3x10^5$ cells) were suspended in complete RPMI-1640 (RPMI 1640 with 20mM HEPES, 10% heat inactivated FBS, 100 IU penicillin/ml, 100 µcells) and left at 39°C in humidified atmosphere at 5% CO_2 for 1h to restore resting conditions. The PMN were then incubated for 1h at 39°C or 41°C in humidified atmosphere of 5% CO_2. The temperature of 41 °C for this and for the experiments described below was selected to simulate conditions of whole body hyperthermia, which can be detected in severely heat stressed dairy cows (Silanikove, 2000). All the experiments were carried out in triplicate. The cells were washed twice with sterile HBSS and fluorescein-labelled *E. coli* bioparticles (Pisani et al., 2009) with a ratio of 42 particles/cell were added; PMN were incubated for 1h at 39°C or 41°C in humidified atmosphere of 5% CO_2. Phagocytosis assay was carried out by measuring the fluorescence of fluorescein-labelled *E. coli* bioparticles. Cells were washed twice with sterile HBSS and incubated with 50 µL trypan blue for 1 min at room temperature to quench non-internalised fluorescent bacteria. The cells were washed twice with HBSS. Fluorescence intensity was measured using a fluorescence plate reader (Fluoroscan Ascent). Laser excitation was set at 485 nm and fluorescence emission was collected using a 530 nm band pass filter.

2.4 *Determination of Reactive Oxygen Species (ROS) production*

The modulation of oxidative burst activity of isolated bovine PMN was studied by cytochrome *c* reduction assay as previously described (Rinaldi et al., 2008). All the experiments were carried out in duplicate. Aliquots of 2 x 10^5 PMN were suspended in 100 µl of HBSS with Mg^{++} and Ca^{++} in two 96-well sterile plates and incubated at 39°C in humidified atmosphere of 5%

143 CO_2 for 1h to restore resting conditions. The PMN were then incubated for 1h at 39°C or 41°C in
144 humidified atmosphere of 5% CO_2. At the end of the incubation period, 10 µl of cytochrome *c* (1
145 mM) and 50 µl of HBSS or PMA (2.5 µg/mL final concentration) were added. The final volume of
146 all wells was adjusted to 200 µl with HBSS. Absorbance was measured on a plate reader (Bio-Tec
147 Instruments Inc., Winooski, VT, USA). The optical density (OD) was measured at a wavelength of
148 550 nm for 210 minutes and at 30 min intervals.

149 2.5 *PMN apoptosis measurement*

150 The concentration of PMN was adjusted to $2x10^6$/ml and 25µl of this solution ($5x10^4$ cells),
151 seeded in two 384 wells black sterile cell culture plates and incubated for 1h at 39°C in humidified
152 atmosphere of 5% CO_2 to restore resting conditions. The PMN were then incubated for 2h at either
153 39°C or 41°C in humidified atmosphere of 5% CO_2. All the experiments were carried out in
154 triplicate. Apoptosis was determined by measuring the activities of two enzymes that play effector
155 roles in cows' apoptosis: caspase-3 and caspase-7 (Ceciliani et al., 2007). Briefly, to each wells
156 containing $5x10^4$ PMN in 25µl was added the same volume of Apo-ONE® Homogeneous Caspase-
157 3/7 Reagent, previously diluted 1:100 in the reaction buffer. Fluorescent intensity was measured
158 using a fluorescence plate reader Fluoroscan Ascent. Laser excitation was set at 485nm and
159 fluorescence emission was collected at 530nm band pass filter.

160 2.6 *Statistical analysis*

161 Statistical analyses were carried out using SPSS 22.0 for Windows (IBM, SPSS Inc, USA).
162 Descriptive statistics of different observed parameters are expressed as the mean (±SEM). For
163 phagocytosis, after assessment of normal distribution of data by Shapiro-Wilk Test, treated samples
164 were compared with control samples through a Student's t-Tests for paired samples. For apoptosis
165 and ROS, since the data were not normally distributed (Shapiro-Wilk Test), they were compared
166 using a GEE (generalized estimating equation) to take account of replications, in which the
167 dependent variables had an inverse Gaussian distribution, and an identity link function was used.

168 The effect of treatment, time of replications and their interaction were assessed. Goodness of fit was
169 assessed using a quasi-likelihood under independence model criterion (QIC). The threshold for
170 statistical significance was considered to be $P<0.05$ (*) or $P<0.01$ (**).
171

172 ## 3. Results

173 ### 3.1 *High temperature reduces the phagocytosis capacity of bovine PMN*

174 To explore the capacity of HS to modulate the bovine PMN phagocytosis, the neutrophils
175 were cultured at 39°C or 41°C. Results show that the phagocytosis ability of PMN to engulf
176 fluorescein labelled *E. coli* particles was affected by the exposure to high temperature: the
177 phagocytosis rate was significantly reduced (decrease of 37%, $P<0.01$) when the cells were
178 incubated for 1 h at 41°C compared to 39°C (Figure 1).

179 ### 3.2 *High temperature attenuates ROS production by resting and PMA-stimulated PMN*

180 In order to study whether HS modulates the generation of extracellular superoxide, PMN
181 were incubated at either 39°C or 41°C in the presence of cytochrome c, the reduction of which
182 occurring specifically in response to the generation of extracellular superoxide anion. Results
183 showed that, starting from 1h after HS, the oxidative burst of PMN, determined by ROS production,
184 was significantly reduced by exposure of the cells at 41°C s compared to 39°C (Figure 2A). In
185 detail the ROS production difference was 2% less after 60 min and the gap increased up to 12%
186 after 210 min. The same experiment was carried out on PMA-stimulated PMN, in order to study
187 whether the oxidative activity is influenced by HS in inflammatory condition (Figure 2B). Results
188 showed that PMN stimulated with PMA for 1h produced higher amount of extracellular ROS as
189 compared with resting PMN at both 39°C or 41°C (Figure 2A and 2B). Furthermore, results
190 confirmed that high temperature (41°C) specifically inhibited PMN ROS production starting from
191 2h after HS (Figure 2B). In details, the ROS production differences between PMN cultured at 39

192 and 41°C were higher than those observed in resting PMN and ranged between 2% at 120 min and
193 21% at 210min.

194 3.3 *High temperature does not affect PMN spontaneous apoptosis*

195 To investigate the apoptosis-modulating activity of HS, the enzymatic activities of caspase-3
196 and caspase-7, the two major executioners of the apoptosis pathway, were measured. Caspase 3/7
197 activity was detected 5 h after PMN exposure at 39°C or 41°C. The results show that the high
198 temperature did not influence the apoptosis rate when cells are exposed at 41°C as compared to
199 39°C (Figure 3).

200

201 **4. Discussion**

202 Results from the present study support the concept that HS, more specifically body
203 hyperthermia consequent to the exposure to high air temperature, is likely to dampen some bovine
204 PMN functions and may thus increase the risk of infections.

205 The findings of this study are in agreement with those already reported for dairy cows or
206 humans. Elvinger et al. (1991) described an impairment of ROS production in PMN exposed in
207 vitro to HS conditions, whereas they did not find any significant effect of HS on phagocytosis.
208 Different experimental conditions (42 instead of 41 °C) and different methods for measuring
209 phagocytosis may partially explain this difference. Furthermore, it has to be noticed that Elvinger
210 and co-workers (1991) cultured PMN at 42 °C, representing a temperature of at least 3 °C above
211 normal body temperature. It must be said that these high temperatures are quite unlikely in under
212 field conditions, and life threatening (Bianca, 1968). More recently, other authors (do Amaral et al.,
213 2011) reported that HS due to lack of cooling decreases ROS production and phagocytosis in
214 neutrophils from transition dairy cows kept in hot environment. The exposure of human PMN to 43
215 °C for 1 hour reduced the net intracellular oxidants production by 46% (Callahan et al., 1999).

Furthermore, both macrophages and PMN isolated from healthy humans and exposed *in vitro* to HS conditions showed a significant reduction of NADPH oxidase-mediated O2- generation (Polla et al., 1995).

The mechanisms behind the impairment of phagocytosis and ROS production observed in PMN exposed to 41 °C have not been investigated in the present study. However, results reported elsewhere for human PMN indicated that their exposure to HS conditions elicits a heat shock response (Babcock and Meyer, 1998; Callahan et al., 1999) and that heat shock proteins may exert an anti-inflammatory action. Results referred to immune cells largely differ from those referred to whole body or other cell types (e.g., erythrocytes), which indicate that high temperature stimulates ROS production with consequent higher risk of oxidative stress (Bernabucci et al., 2002; Slimen et al., 2014). The reasons beyond such different behaviour of cells belonging to different compartments are not easy to explain and are beyond the scope of this study.

The anti-inflammatory action of HS has been recently described using an inflammatory model in pig and has been associated with physiological adaptation to high temperature (Campos et al., 2014). Decline of ROS production is also in line with previous results relative to bovine PBMC, indicating a reduction of intracellular ROS at 41 °C incubation temperature as a sign of reduction of cellular metabolic rate (Lacetera et al., 2006). Finally, the decline of ROS production in PMN at high temperatures may represent an adaptive mechanisms which reduce the risk of oxidative stress commonly observed in heat stressed dairy cows (Bernabucci et al., 2002).

The fine tuning of PMN's lifespan in tissues is essential to reduce inflammatory-derived injuries to the host (Greenlee-Wacker, 2016). We can rule out that the downregulation of ROS production and the decrease of phagocytosis is related to a decrease of cells' number due to apoptosis, given that the apoptosis rate of bovine PMN was not found to be affected by HS. Available data on the effects of HS on apoptosis of human PMN are conflicting, depending on the temperature of HS and time of exposure. Nagarsekar and co-workers (2008) found that culturing

241 human PMN at 39.5 °C for 8h greatly accelerated caspase dependent apoptosis. Exposure of human
242 PMN to 43 °C for 1 hour caused an early increase in the rate of apoptosis (Callahan et al., 1999),
243 whereas in a recent in vitro study carried out by culturing human neutrophils at 37 or 43 °C for 10
244 minutes, no substantial differences in viability were observed (Boyko et al., 2014). Therefore, we
245 may hypothesize that the temperature effects depend on the length of exposure.
246

247 ## 5. Conclusions

248 In synthesis, the results of the present in vitro study suggest that some functions of PMN
249 exposed to temperature simulating conditions of severe whole body hyperthermia are impaired and
250 may at least partially explain the higher occurrence of infections during periods of hot weather.

251 Further epidemiology studies are necessary to clarify whether and under which specific
252 circumstances high environmental temperatures are associated with higher incidence of infections.
253 However, the risk of impairment of PMN functions under these conditions support the use of
254 management practices (i.e. cooling), which may help to limit the increase of body temperature and
255 optimization of other environmental features (i.e. general hygienic conditions, nutritional plans,
256 etc.), which may also play a role in causing infections outbreaks in hot environments.
257

258 ## Abbreviations

259 **HS**: heat stress; **PBMC**: peripheral blood mononuclear cells; **PMN**: polymorphonuclear cells; TNF-
260 **alpha**: tumor necrosis factor alpha; **INF-gamma**: interferon gamma; **PMA**: phorbol 12-myristate
261 13-acetate; **ROS**: Reactive Oxygen Species; **HBSS**: Hank's Balanced Salt Solution; **OD**: optical
262 density.

263 ## Acknowledgements

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Competing interest

The authors declare that they have no competing interests.

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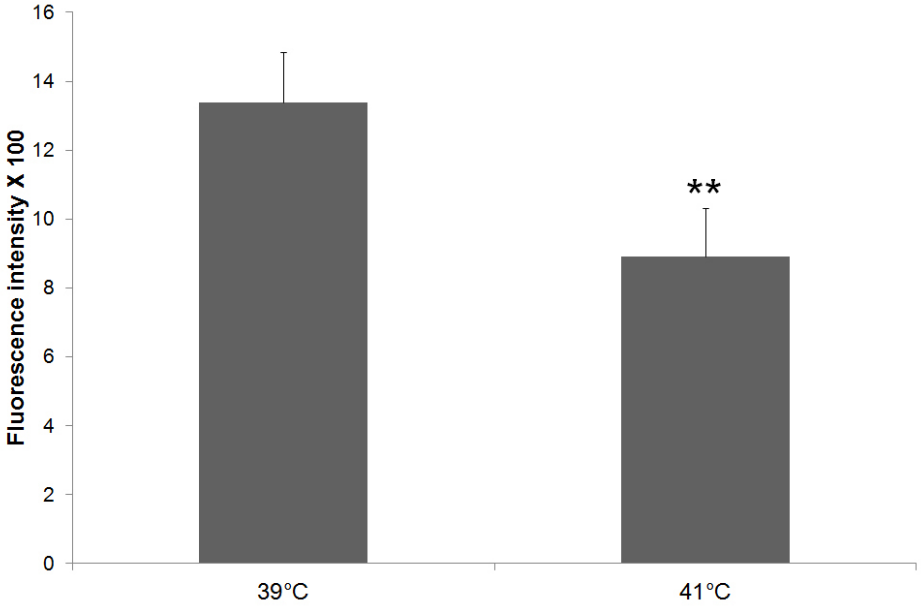
362 **Figure legends**



363

364

365 Fig.1. Phagocytosis of fluorescein labelled *E. coli* bioparticles by bovine PMN.

366 The cells were pre-incubated at 39°C or 41°C and after 1h the *E.coli* bioparticles were added.
367 Phagocytosis was allowed to proceed for 1h at 39°C or 41°C. Each assay was carried out in
368 triplicate. Data are means ± SEM of seven independent experiments. Significance was declared for
369 $P<0.05$ (*) and $P< 0.01$ (**).

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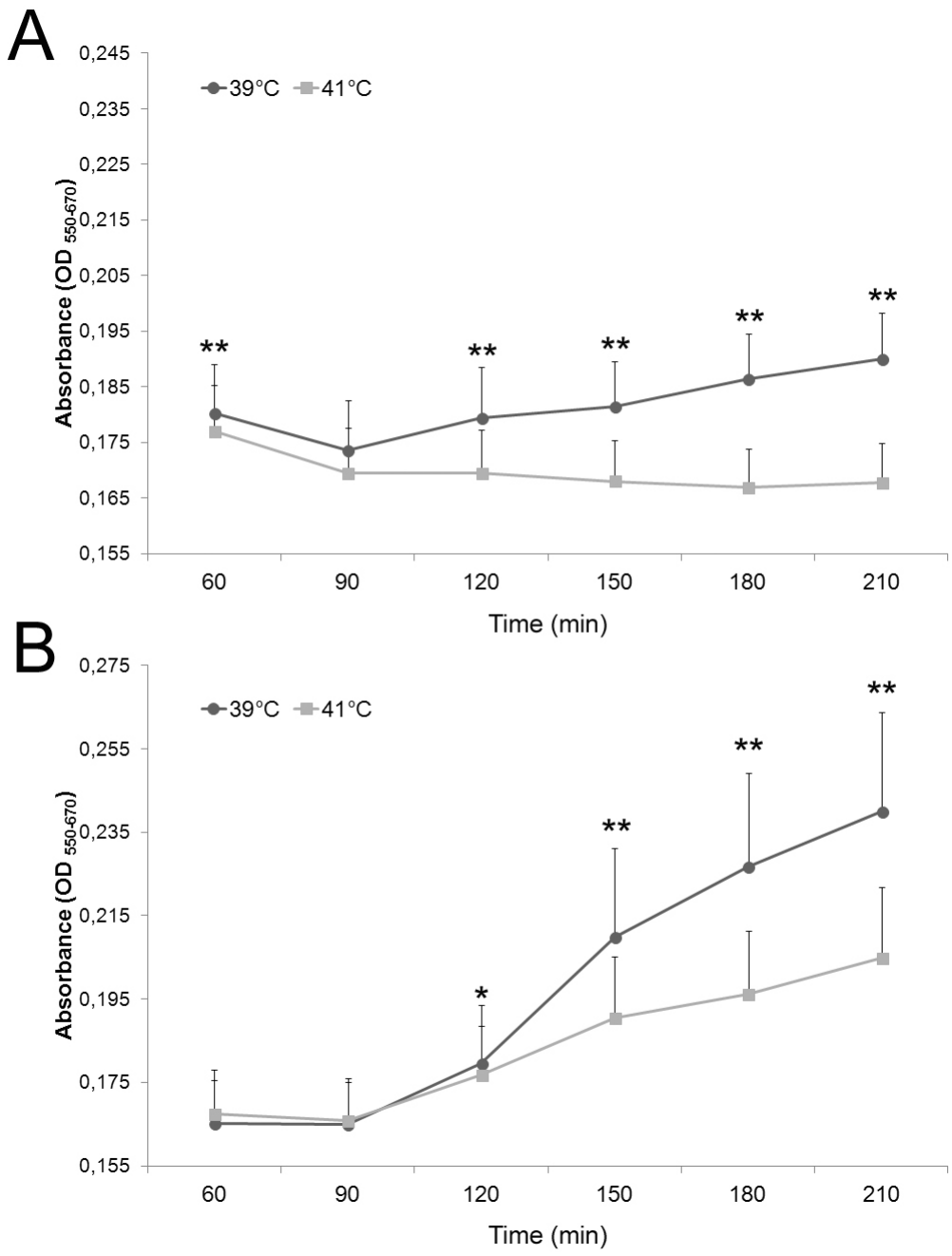
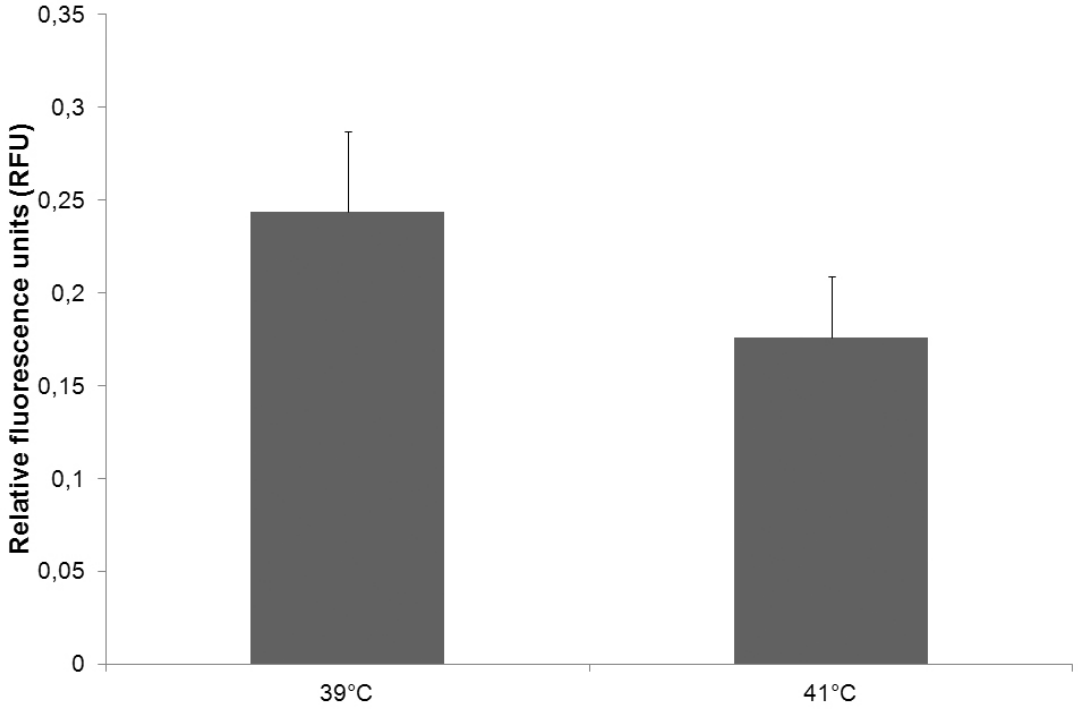


372

373 Fig.2. Effect high temperature on bovine PMN generation of extracellular superoxide as measured
374 by cytochrome c reduction.

375 Resting (A) or PMA-stimulated (B) PMN were exposed to 39°C or 41°C in the presence of
376 cytochrome c and absorbance values measured every 30 min. Each assay was carried out in
377 duplicate. Data are means ± SEM of seven independent experiments. Significance was declared for
378 $P<0.05$ (*) and $P< 0.01$ (**).



379

380 Fig.3. The caspase 3/7 enzymatic activity (spontaneous apoptosis) of bovine PMN.

381 PMN were incubation at 39°C or 41°C for 5 h. Each assay was carried out in triplicate. Data are

382 means ± SEM of seven independent experiments.

383

384