

# **Quantifying Transient Psychological Stress Using a Novel Technique: Changes to PMA-Induced Leukocyte Production of ROS In Vitro**

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*Although much work has been conducted to quantify the long-term physiological effects of psychological stress, measures of short-term, low-level stress have been more elusive. This study assessed the effect of exposure of volunteers to a mild, brief, psychologically stressful event, on the functional ability of leukocytes in whole blood to respond to phorbol 12-myristate 13-acetate (PMA) in vitro. Volunteers operated a car electric window and adjusted it to 4 pre-determined positions. Between each operation the mechanism's polarity was covertly altered (group B) or remained unaltered (group A). For each treatment group 10 different subjects provided capillary blood samples pre- and post-stressor. Using a chemiluminescent technique termed leukocyte coping capacity, the ability of leukocytes to produce reactive oxygen species (ROS) in vitro was assessed. ROS release differed significantly at 10 min post-stressor between treatment groups, suggesting exposure to acute psychological stress leads to a reduced ability to respond to bacterial challenge.*

chemiluminescence   humans   leukocytes   luminol   momentary confusion   psychological  
stress   reactive oxygen species

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## **1. INTRODUCTION**

Psychological stress—a threat which would not require a physiological response which elicits

physiological consequences [1]—has been reported to reduce the effectiveness of the immune system, thus leading to an increased risk of infection or disease [2, 3, 4]. Even short-

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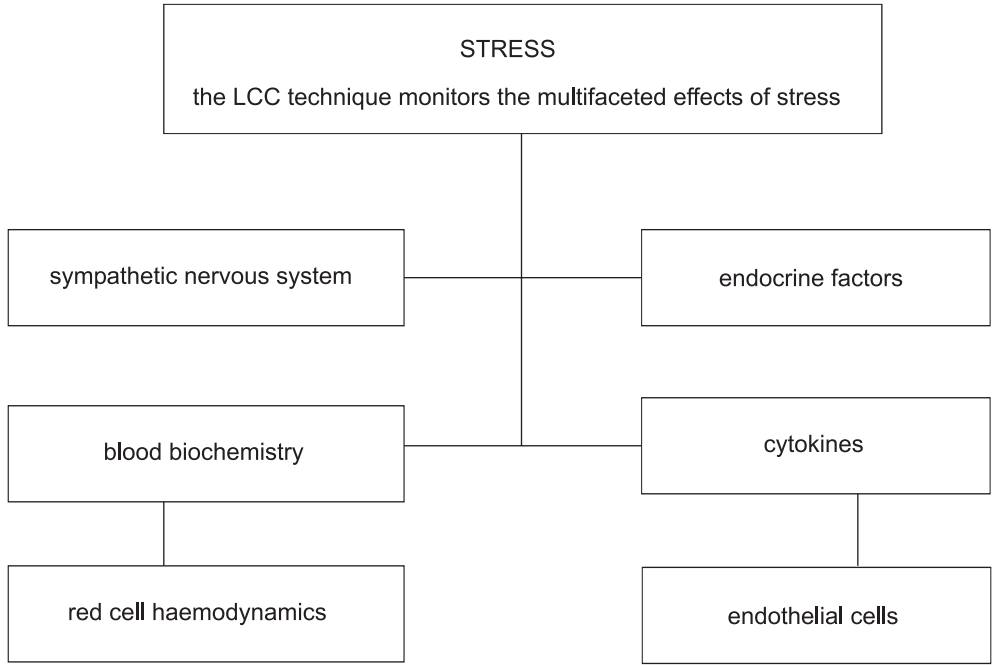
term psychological stressors such as academic examinations [5, 6] can produce demonstrable physiological changes in heart rate (HR), blood pressure (BP) and the activation of specific classes of leukocyte, notably neutrophils [7, 8]. Evidence also exists for an immune-enhancing effect during acute physiological and psychological stress [9, 10]. Activated neutrophils release an array of mediators including reactive oxygen species (ROS) [11], which in addition to their function in attacking invading pathogens, also have the potential to damage healthy tissue and organs [1, 12, 13].

Epidemiological studies indicate that individuals who experience greater degrees of psychological stress are more susceptible to opportunistic infections [14, 15]. Clover, Abell, Becker, et al. linked stress associated with an unstable family environment to an increased incidence of upper respiratory tract infection and influenza B [15]. Similarly, accumulating stress has been linked to chronic reduced immune-competency and increased susceptibility to opportunistic infections in elite athletes [16]. Rodriguez-Galan, Correa, Cejas, et al. demonstrated how the opportunistic fungal disease *Candida albicans* proliferated in stressed rather than non-stressed individuals

[17]. These studies and others [8, 9, 10, 18, 19] provide evidence to suggest that exposure to acute psychological stressors, even for limited periods, can result in an increase in the number of circulating leukocytes.

Many everyday stressors are subliminal in nature, prompting the question of whether they may have immunological consequences. Furthermore, these subliminal stressors may be altering the function of the human immune system and the ability to combat other infections, effects which might be detected and measured through an alteration in leukocyte responsiveness. As an example, we have investigated whether changes in leukocyte responsiveness can be detected in people faced with altered functionality of a basic motor vehicle control.

Due to their systemic distribution and responsiveness to the numerous signals of stress (Figure 1), the leukocyte coping capacity (LCC) test utilises the body's leukocytes (primarily, but not exclusively neutrophils) to provide a bio-indication of the multifaceted effects of stress [7]. Leukocytes (primarily, but not exclusively, neutrophils) have over 250 different receptors [20] which can respond to a diverse range of factors, all of which are sensitive to



**Figure 1. Flow diagram showing factors believed to affect the activation state of leukocytes.** Notes. LCC—leukocyte coping capacity. This figure was first published in Shelton-Rayner, Macdonald, Chandler, et al. [7].

stress. These include endocrine factors in the plasma, cytokines and factors released from other cells, both circulating and non-circulating cells such as endothelial cells, changes in erythrocyte haemodynamics (the changes in membrane deformability of red cells which can alter the sheer stress and viscosity of the blood resulting in altered interactions with leukocytes and the endothelium [21]), changes in blood biochemistry, and changes in the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system. The constant exposure to each of these stimuli pertains to their effectiveness as stress indicators. LCC, i.e., their ability to respond to an external stimulator and produce reactive oxygen species, will be affected by the immediate external environment in the blood. Leukocytes (mainly neutrophils) which have been exposed to stressors within the body will have a reduced capacity to produce reactive oxygen species in response to an external stimulator [20].

Leukocytes are three-dimensional entities. Their ability to produce reactive oxygen species is altered by cell signalling pathways of other entities and cells [20]. The LCC test provides a physiologically relevant means for monitoring the cellular capacity of leukocytes to produce superoxide radicals in real time. The physiological relevance is convincing since leukocytes remain suspended in whole blood which permits dynamic three-dimensional interaction with surrounding erythrocytes and cell-cell interaction within and between different leukocyte cohorts; both have the potential to dramatically affect leukocyte responsiveness. Interaction and exposure to other leukocytes, hormones and cytokines released from surrounding cells can affect leukocyte responsiveness via altered shear stress and expression of cell surface receptors. As cellular integrity is maintained, the potential disruption to cell signalling pathways is limited. The LCC technique also avoids centrifugation, a process known to affect cell reactivity, and also “plating out” cells on glass slides—as used in the nitroblue tetrazolium (NBT) test [22]. The cells are stimulated *in vitro* with phorbol 12-myristate

13-acetate (PMA) and their superoxide producing capacity is measured in real time. As leukocytes release reactive oxygen species in response to stress [23], the stimulation allows us to evaluate the leukocyte’s (predominantly, but not exclusively neutrophils) capacity to generate further reactive oxygen species. This takes into account the exposure to other stress mediators and makes the test sensitive to true stress; the reactivity of the cells is not altered by deliberate manipulation.

Utilising the LCC technique for the quantification of leukocyte activity we investigated whether exposure to a psychological stressor, lasting only seconds and at a magnitude which might normally be dismissed as trifling, could be detected in terms of changed leukocyte activation in otherwise healthy subjects.

## 2. MATERIALS AND METHODS

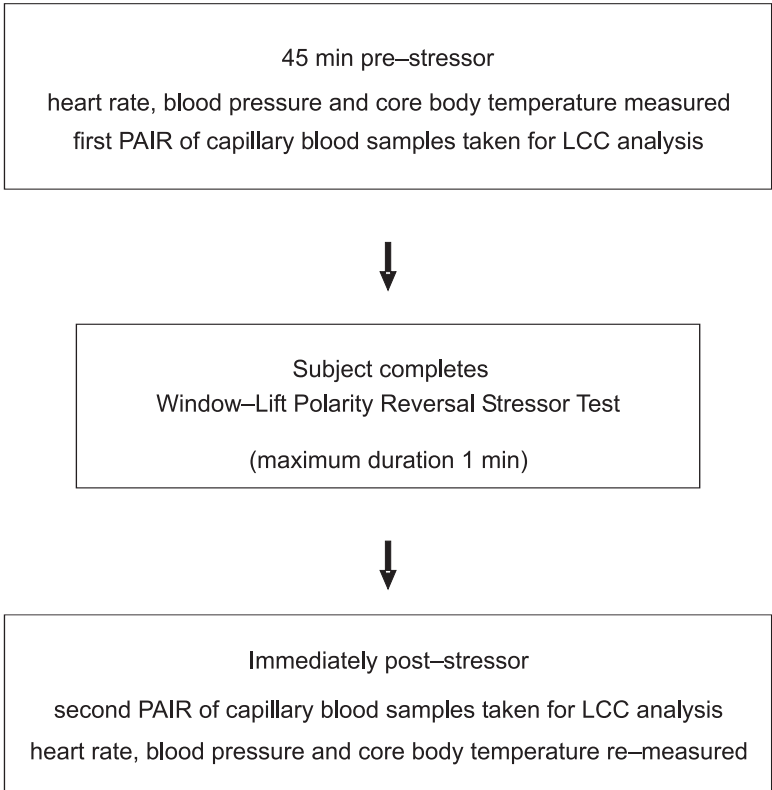
### 2.1. Subjects

Local ethical committee approval from Coventry University Ethics Committee and informed consent was obtained before commencement of the study, in accordance with the declaration of Helsinki [24].

Ten male and 10 female healthy individuals, aged between 27 and 53 years, were recruited from a pool of 40 volunteers. Volunteers were excluded if they suffered from psychiatric illness, respiratory or cardiovascular disease, were smokers, had taken prescription medicine within the previous month, or if they possessed prior knowledge of the test equipment.

### 2.2. Design

The subjects were assigned to one of two treatment groups, group A (control) and group B (intervention). Both groups contained equal numbers of each gender. The experimental protocols were rigorously standardised, and testing was confined to between the hours of 10:30 and 15:00. Subjects were required to avoid any strenuous activity for at least 2 h prior to testing (e.g., they were instructed to take the lift to the laboratory, rather than climb the stairs).



**Figure 2. Flow diagram illustrating the time line for blood sample collection, for leukocyte coping capacity (LCC) analysis, and measurement of other physiological parameters (heart rate, systolic and diastolic blood pressure and core body temperature).** *Notes.* This figure was first published in Shelton-Rayner et al. [7].

Prior to obtaining resting HR, BP and core body temperature (CBT) following the standardised procedure outlined in this section and illustrated in Figure 2, subjects sat quietly and were instructed to breathe orthonasally for 15 min. The first pair of capillary blood samples was then taken 45 min before exposure to the test apparatus (45 min pre-stressor).

During the 45-min pre-stressor period subjects were asked to sit quietly or read (reading material was of a non-stimulating content, a local daily newspaper).

Approximately 2 min before the start of the trial, the subject was ushered to an isolated, previously unseen area of the lab, where they were seated comfortably and the test protocol explained. The test lasted a maximum of one minute, during which time the subject was requested to open a car electric window to a designated level. After a 5-s pause the subject was then to open the window fully, then, after a further 5 s, partially closing the window to the designated halfway mark before,

after a final 5-s pause, closing the window fully. It was explained that once the test had begun no further verbal communication was allowed, although the researcher would state when each 5-s pause had elapsed.

For the test, a Jaguar X-Type passenger door was secured in a wooden stand simulating the correct height and position a passenger would experience within a normal car. The electric window was powered with a 20 V DC power supply. The door had been retro-fitted with a switch which allowed the researcher to alter, unseen, the polarity of the electric window mechanism, so that when the subject pressed the up-button the window lowered and vice versa. The door was unveiled to the subject immediately before the test started.

During the test, the function of the window mechanism was as indicated by the arrows on the switch for group A (control); however, the polarity of the window was reversed after each 5-s pause for group B (intervention).

Immediately upon completion of the task HR, BP and CBT were recorded and further blood samples taken (Figure 2).

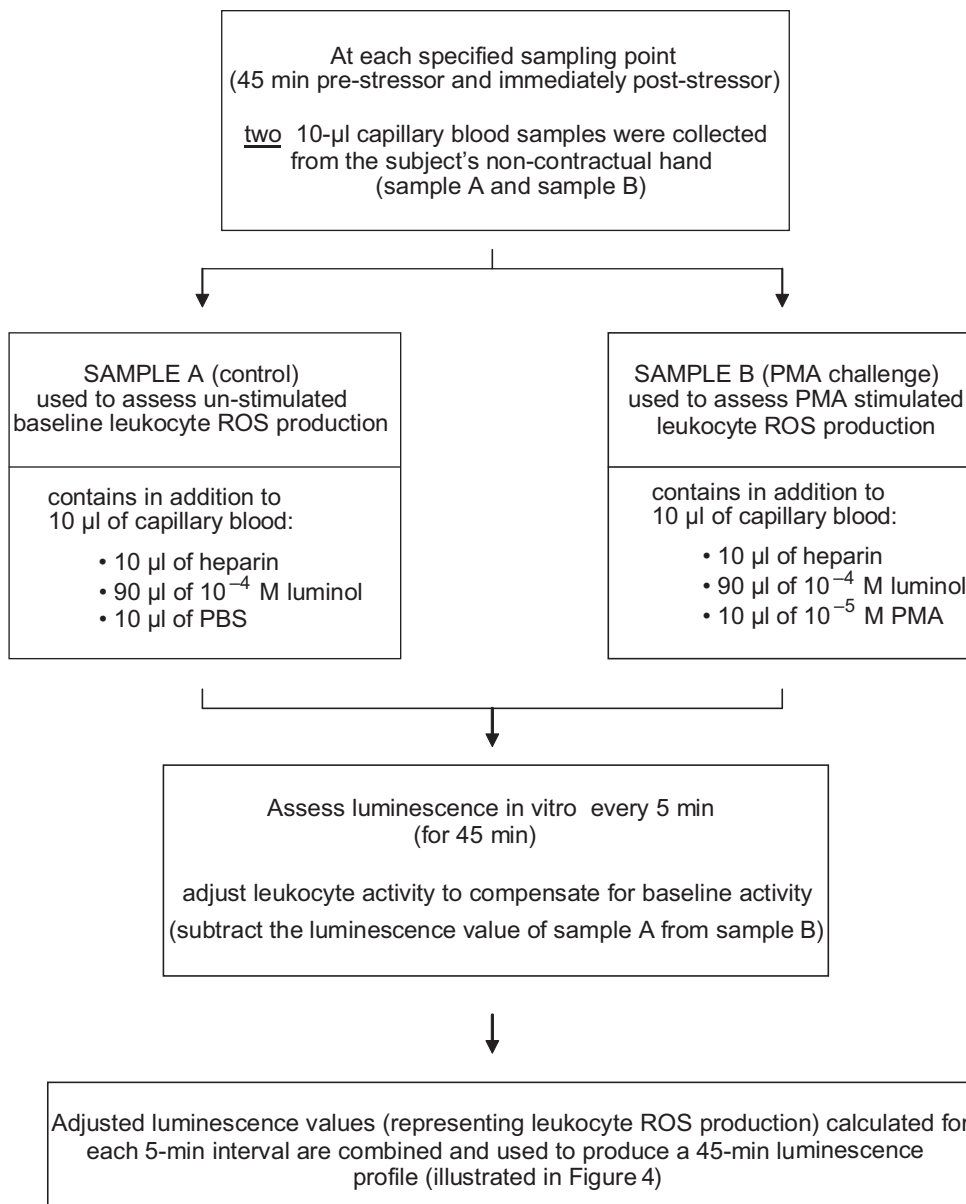
### 2.3. HR, BP and CBT Measurements

At each specified time point (Figure 2) a heart rate recorder (Polar 610i™ heart rate monitor, Polar Electro, Finland) attached directly to the chest, using the belt provided, monitored HR. Systemic BP was measured using an

oscillometric wrist mounted blood pressure monitor (Omron RX-3, Omron Healthcare, USA). CBT was measured using an infrared ear thermometer (Braun® Thermoscan™, P&G Brooklands, UK).

### 2.4. Blood Samples

At each specified time point (Figure 2) two 10-µl blood samples were taken using a finger lancing device (Accu-Chek® Softclix®, Roche®,



**Figure 3. Leukocyte activity was assessed using whole blood samples taken 45 min pre- and immediately post-stressor, following the protocol illustrated.** Notes. PMA—phorbol 12-myristate 13-acetate, ROS—reactive oxygen species, PBS—phosphate buffered saline.

UK) from the subject's non-contractual hand. Following the procedure illustrated in Figure 3, one sample was used for the (non-stimulated) control (sample A) and was placed into 10  $\mu$ l of murine heparin (concentration 0.1 units) (CP Pharmaceuticals, UK), 10  $\mu$ l phosphate buffered saline (PBS) (Sigma Aldrich, UK) and 90  $\mu$ l of  $10^{-4}$ M luminol ( $C_8H_7N_3O_2$ ) (Sigma Aldrich, UK). The second blood sample (sample B) was added to the same reagents, except that the 10  $\mu$ l of PBS was replaced with 10  $\mu$ l of  $10^{-5}$ M PMA (Sigma Aldrich, UK). PMA stimulates leukocytes (primarily neutrophils) causing them to increase their production of oxidative metabolites. This increased production can be measured using luminol amplified light emission (chemiluminescence) [25].

## 2.5. Determining Leukocyte Activity

LCC is a measure, made using a luminometer, of the concentration of ROS calibrated through the emission of photons as a result of their interaction with luminol. This is an indicator of the leukocytes' ability to produce a respiratory burst [23]. LCC is defined as the response of leukocytes (mainly neutrophils) to challenge, in this case by PMA. Subjects whose LCC score is higher have displayed a greater potential to produce a respiratory burst, and are therefore in this respect more able, physiologically, to respond to bacterial challenge (immunologically-competent). LCC responsiveness to in vitro PMA challenge is inversely related to stress level.

In this experiment, each pair of blood solutions (sample A and sample B) was simultaneously tested every 5 min using a luminometer (Berthold® Technologies, Junior™ LB9509, UK) for a total of 45 min (Figure 3), to produce a luminescence profile (Figure 4). Between chemiluminescence measurements the samples were incubated at 37 °C in a water bath (JB1™ Grant Instruments, UK). At each 5-min interval an adjusted score, measured in relative light units ( $RLU_{adj}$ ) was obtained for each subject by subtracting the luminescence score of the control (sample A, without PMA stimulation) from the PMA challenge sample (sample B).

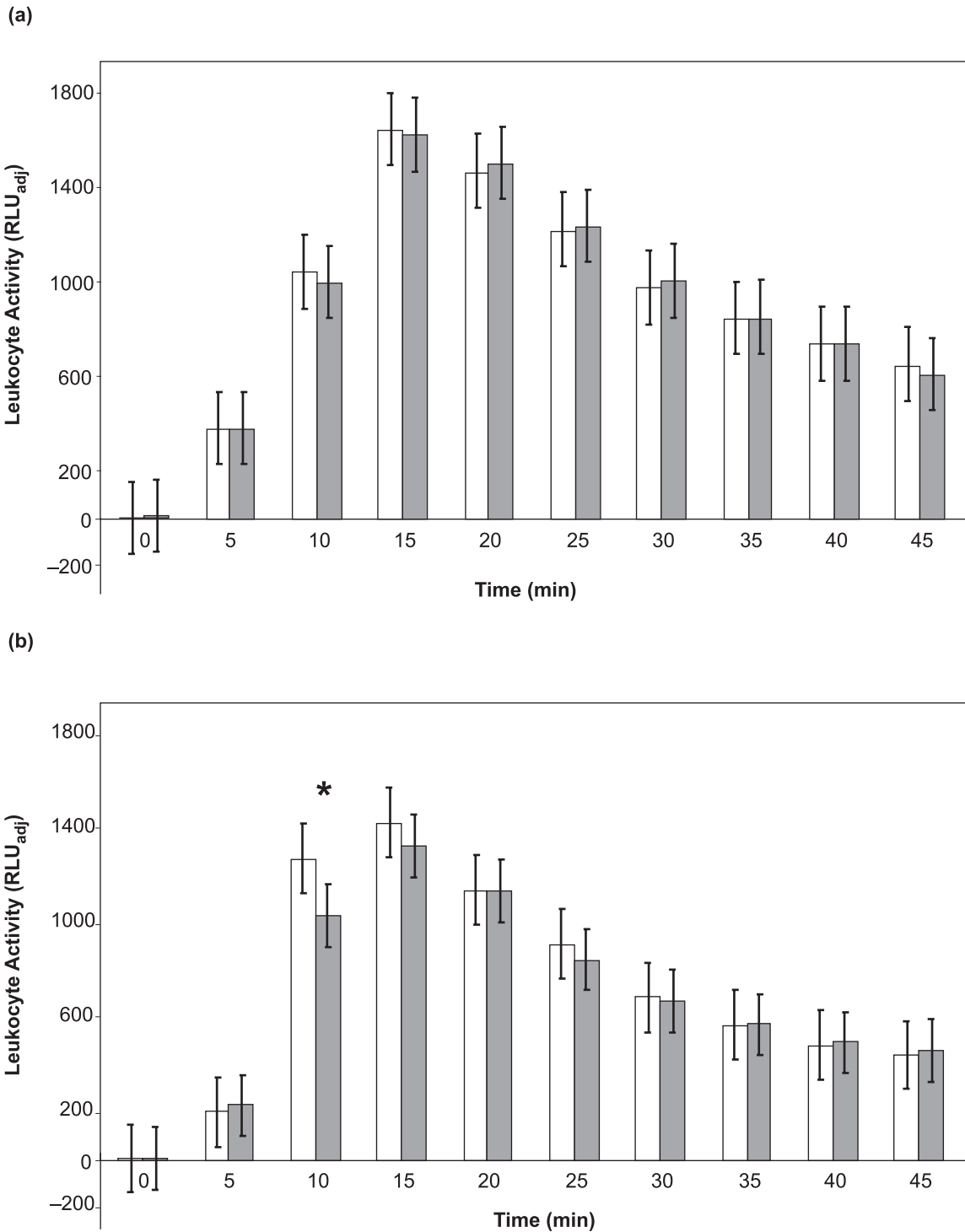
## 2.6. Data Analysis

For all measured parameters data are expressed as mean post-stressor changes  $\pm$  standard error of mean (*SEM*). For  $T_{max}$  (time taken to reach maximum leukocyte activity), the data were classed as discontinuous as leukocyte activity was measured at 5-min intervals for a total of 45 min, in this case the median  $\pm$  *SEM* is presented. Single factor analysis of variance (ANOVA) (SPSS version 15.0) was used firstly, to test in turn, the effect of treatment group on post-stressor changes in leukocyte activity and also HR, CBT and systolic and diastolic BP. Secondly, to explore, in isolation, the effect of the putative stressor on each treatment group by comparing pre- and post-stressor leukocyte activity for each response attribute (e.g., maximum adjusted leukocyte activity,  $Hmax-RLU_{adj}$ , and adjusted activity at 5, 10 and 15 min into the 45-min luminescence profile), in addition to each of the other assessed physiological parameters (HR, BP and CBT).

## 3. RESULTS

### 3.1. Leukocyte Activity

LCC profiles are displayed in Figure 4, with post-stressor changes in activity for the assessed attributes of the luminescence profiles given in Figure 5. Data are expressed as mean differences between leukocyte activity at 45 min pre- and immediately post-stressor ( $RLU_{adj}$ )  $\pm$  *SEM*. Following the test, the mean change in LCC response ( $Hmax-RLU_{adj}$  and  $T = 10$  min) between 45 min pre- and immediately post-stressor for group B was greater than that of group A (control) (Figure 5). In general, LCC scores were depressed following the test in group B, but not in group A (Figure 4). Most strikingly, at  $T = 10$  min the post-stressor change in leukocyte activity for the control (group A) exhibited a decrease of  $46.2 \pm 38.2 RLU_{adj}$  which was significantly different to the larger decrease observed within group B ( $240.0 \pm 56.1 RLU_{adj}$ ) ( $F_{1,19} = 8.23$ ,  $P = .001$ ). A similar trend was noted at maximum leukocyte activity ( $Hmax-RLU_{adj}$ ) where group A demonstrated a post-stressor decrease of  $41.6 \pm$

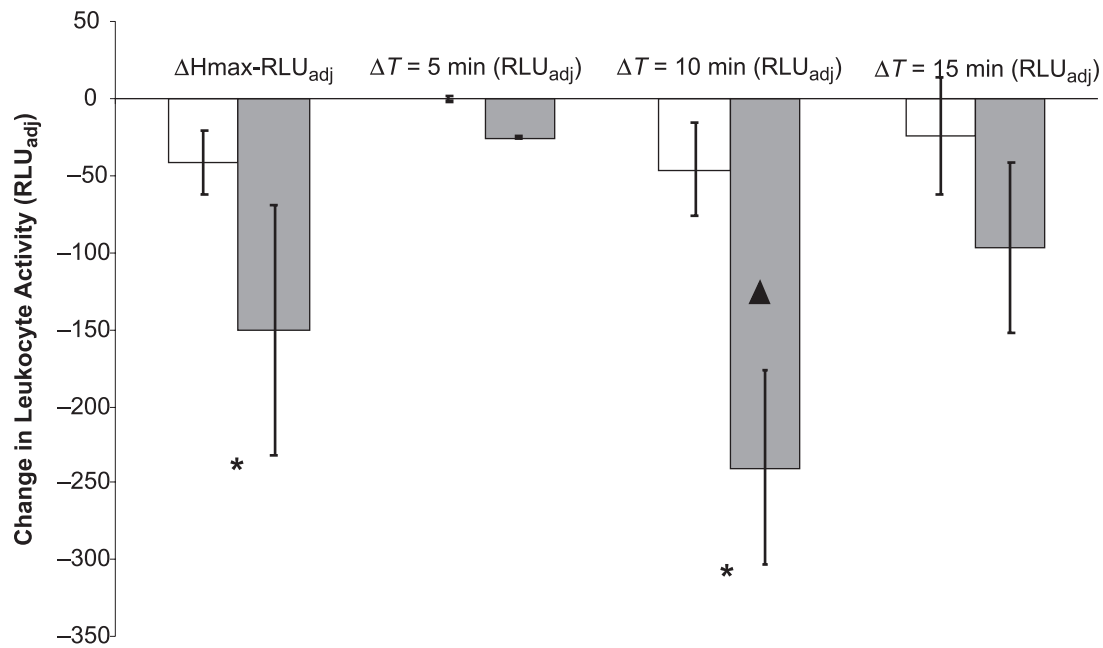


**Figure 4. Mean adjusted leukocyte coping capacity (RLU<sub>adj</sub>)  $\pm$  SEM for (a) treatment group A and (b) treatment group B ( $n = 10$  for each). Notes.** Open bars represents mean adjusted leukocyte activity 45 min pre-stressor and closed bars represents activity immediately post-stressor. \*—significant difference in activity between 45 min pre- and immediately post-stressor ( $P < .05$ ).

20.7 RLU<sub>adj</sub>, compared to a decrease between pre- and immediately post-stressor for group B ( $150.7 \pm 82.0$  RLU<sub>adj</sub>) ( $F_{1,19} = 5.3$ ,  $P = .04$ ). No significant post-stressor differences were recorded

for the remaining parameters of the leukocyte luminescence profiles ( $T_{\max}$ ,  $T = 5$  min and  $T = 15$  min) between 45 min pre- and immediately post-stressor or between treatment groups.





**Figure 5. Effect of stressor on leukocyte activity.** Notes. Mean and standard error of the mean (SEM) are presented for the change ( $\Delta$ ) in leukocyte activity (difference between 45 min pre- and immediately post-stressor samples) for 4 specific attributes of the leukocyte luminescence profiles for each treatment group ( $n = 10$  for each) (leukocyte activity, adjusted relative light units,  $RLU_{adj}$ ), open bars represent group A (control) and closed bars group B (intervention).  $H_{max}$  ( $RLU_{adj}$ )—the maximum adjusted response exhibited during the 45-min sampling period,  $T = 5, 10$  and  $15 \text{ min}$ —the adjusted response in leukocyte activity recorded at 5, 10 and 15 min into the 45-min activity profile ( $RLU_{adj}$ ). Single factor ANOVA was used to investigate the effect of treatment on leukocyte activity ( $df = 19$ ). ▲—difference between pre- and post-stressor leukocyte activity ( $P < 0.05$ ), \*—difference between group A and group B ( $P < 0.05$ ).

### 3.2. CBT

No significant post-stressor differences in CBT between 45 min pre- and immediately post-stressor or between treatment groups were recorded.

### 3.3. HR, BP

There were no significant post-stressor differences in either HR or systolic or diastolic BP between 45 min pre- and immediately post-stressor, or between treatment groups.

## 4. DISCUSSION

Exposure to subtle and transient psychological stressors—essentially little more than a moment of confusion—elicited a quantifiable change in leukocyte responsiveness to PMA stimulated production of ROS in vitro. The results demonstrate that the ability of leukocytes

to produce ROS and subsequently to kill bacteria is reduced immediately after even mild psychological stress. This effect is transient, with leukocytes recovering the capacity to produce ROS within 45 min. This response is comparable in magnitude to that observed in our previous study, where a stressor of greater duration and intensity was assessed using the same methodology [7]. In that instance the stressor lasted 6 min and involved the use of an automotive touch screen interface to complete three tasks (such as programming a destination into a satellite navigation system). At  $T = 10 \text{ min}$  the post-stressor decrease in leukocyte activity observed by Shelton-Rayner et al. [7] following a stressor lasting 6 min was  $322.0 \pm 138.6 RLU_{adj}$ , whereas for the current study group B showed a post-stressor decrease of  $240.0 \pm 56.1 RLU_{adj}$ . Preliminary studies with longer term stressors (such as divorce and bereavement) indicate that longer term stressors have a sustained



effect on the ability of leukocytes to produce ROS, with recovery taking months [26]. It has long been accepted that physiological stressors such as exercise and excess physical exertion can influence aspects of the immune response; specifically leukocytosis from marginal pools [27, 28]. Furthermore, exposure to hostile conditions or other psychological stressors initiates the secretion of several hormones, including cortisol, catecholamines, prolactin, oxytocin and renin [29, 30, 31, 32], any of which could lead to the alteration of adhesion receptors on circulating leukocytes and thus alter their distribution and activation state.

Neutrophils can respond rapidly to a wide range of physical and psychological stressors, and these responses can affect the ability of the immune system to react to ongoing or potential challenge [6, 16, 33]. Stress has been shown to influence the number, distribution and activation state of neutrophils in the blood in a rapid and reversible manner [7, 8, 23, 26, 33, 34]. Although each of the cited examples describes the use of an acute stressor, all were of far greater intensity and duration compared to the current stressor paradigm. Against this background, ours is the first study of which we are aware to demonstrate that a transient psychological stressor lasting only a few seconds significantly affects the ability of leukocytes in whole blood to produce ROS *in vitro* in humans. Leukocytes are three-dimensional entities. Their ability to produce reactive oxygen species is altered by cell signalling pathways of other entities and cells.

Leukocytes (primarily, but not exclusively, neutrophils) possess in excess of 250 different receptors capable of responding to a diverse range of factors, e.g., altered blood biochemistry and erythrocyte haemodynamics, endocrine factors in the plasma, cytokines and other factors released from both circulating and non-circulating cells including endothelial cells, and changes in the hypothalamic-pituitary-adrenal axis and sympathetic nervous system (illustrated in Figure 1); all of which are sensitive to stress [20]. The constant exposure of leukocytes to each of these stress stimuli makes them ideal bio-indicators for the presence and magnitude

of stress. LCC, i.e., their ability to respond to an external stimulator and produce reactive oxygen species, will be affected by the immediate external environment in the blood [7].

The architecture and adhesiveness of a cell microenvironment is essential for the effective determination of its ability to respond *in vivo* [35]. The deliberate suspension of leukocytes in whole blood during LCC analysis, ensures that the structural integrity and morphology of the cell remains as near to the *in vivo* condition as possible. It also permits the dynamic interaction with surrounding erythrocytes and allows cell-cell interaction within and between different leukocyte cohorts [7]. During LCC analysis the process of centrifugation and plating out cells on glass slides (used in the NBT test) [22] are both avoided, as both can potentially reduce cellular responsiveness via disruption to cellular integrity and signalling pathways. The process allows the leukocytes (primarily neutrophils) to interact with hormones (which can alter the reactivity of the cells), other cells such as macrophages, other neutrophils, the haematocrit, and red blood cells (whose viscosities alter during stress) [7].

This study demonstrates that the LCC technique is sensitive enough to detect the subtle change in immune-competency manifested as a result of exposure to a transient situation designed to cause psychological confusion. A significant difference in leukocyte activity following exposure to the stressor was demonstrated between treatment groups, with post-stressor LCC scores generally being depressed in group B, but not in group A (Figures 4–5). Despite confirmation of the presence of psychological stress, no significant post-stressor differences in HR, systolic and diastolic BP were recorded between treatment groups. This would suggest that the innate immune system (specifically leukocytes) possesses a greater sensitivity to subtle changes in emotional and psychological status, when compared to cardiovascular responsiveness. This would imply that its assessment should be used in preference to more traditional measures as a means of detecting the presence of, and quantifying, increased mental loading and psychological stress.

In conclusion, this research is the first reported study on the effect of an acute short-term psychological stressor (lasting only seconds) on polymorphonuclear leukocyte activation. It was observed that exposure to a psychological stressor, lasting only seconds, was sufficient to reduce leukocyte responsiveness to an external stimuli. This study has demonstrated that leukocytes are extremely sensitive to any changes in physical and mental stress level. This technique provides the potential means for objectively capturing the effects of even mild transient stressors.

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