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Leukocyte Responsiveness, a Quantitative Assay for Subjective Mental Workload.

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ABSTRACT

Assessing psychological stress and mental workload within work-based scenarios relies heavily upon qualitative, subjective, self-assessment techniques, many of which were originally intended for identifying specific pathological disorders and have reduced sensitivity when evaluating everyday stressors. Quantitative measures involve monitoring changes in the cardiopulmonary system and stress hormone concentration. Although these (e.g. heart rate and blood pressure) provide a basic, reactive indication of the presence of a psychological stressor, many are subject to influence by other bio-mechanisms, or are unable to provide rapid results due to complex laboratory analysis. This study demonstrates how immune responsiveness, known to be influenced by psychological stress, can be used to assess changes in mental workload. Healthy male and female subjects (aged between 26 and 55 years) provided capillary blood samples before and after completing the same, basic, driver-related tasks followed by a simple manoeuvre in two unfamiliar motor vehicles. Using a chemiluminescent technique termed Leukocyte Coping Capacity (LCC), the ability of leukocytes to produce reactive oxygen species *in vitro* was assessed. Significant post-stressor changes in leukocyte activity were demonstrated between treatment groups. These findings add weight to the proposition that leukocyte activation is a useful quantitative measure of psychological stress and mental loading in humans. This study demonstrates the diagnostic ability of LCC for use during ergonomic evaluation, however the potential industrial applications for this technique are numerous and diverse.

KEY WORDS

ABBREVIATIONS

Hmax-RLU$_{adj}$, maximum control adjusted leukocyte activity; **LCC**, leukocyte coping capacity; **RLU$_{adj}$**, control adjusted relative light units; **T-max**, time taken (minutes) to reach maximum control adjusted leukocyte activity; **T=5, 10, and 15minutes**, control adjusted leukocyte activity at 5, 10, and 15 minutes, respectively, into the 45 minute chemiluminescence sampling protocol.

1.0   INTRODUCTION

Evaluation of psychological stress – a threat which would not require a physiological response which elicits physiological consequences (Segerstrom and Miller, 2004) relies heavily upon qualitative psychometric measures. Examples include: Present State Examination (Baker et al., 2003), the Brief Symptom Inventory (Brosig et al., 2007; Cuculi et al., 2006; Fellinger et al., 2007; Mansbach et al., 2005), and the Buss-Durkee Hostility Inventory (Bag et al., 2005). All were primarily designed to identify specific pathologic disorders, and validated using dysfunctional clinical populations with abnormal statistical distributions. Consequently these proved to have limited sensitivity when used to test below intended critical diagnostic thresholds (Lemyre and Tessier, 1988) – such as when investigating psychological and physiological transient laboratory stressors.

As psychological stress attained social acceptance, the need for reliable dedicated measurement techniques led to the development of diagnostic systems, most recently the Psychological Stress Measure – used to evaluate health and wellbeing in the workplace (Lemyre and Tessier, 2003). The subjective workload assessment technique and the NASA-task load index evaluate alterations in perceived mental workload, and are utilised during the design process of new technology to assess ergonomic impact (Syroid et al., 2002). Although these systems provide an insight

into how an individual reacts to potentially stressful situations, their conclusions are subjective. There is therefore a need for accurate quantitative assessment techniques.

Quantitative measures of psychological stress focus primarily upon monitoring characteristics of the cardiopulmonary system and assay of specific stress hormones, including salivary cortisol (Clow et al., 2010, 2006; Pfeffer et al., 2009; Powers et al., 2006; Stalder et al., 2011) and catecholamines (Brown et al., 2003). Despite the fact that hormone analysis requires several hours before results are known, studies have demonstrated how techniques, for example monitoring urinary catecholamine excretion, can be used as an objective means of quantifying the effects of long-term psychological stressors (Brown et al., 2006 and 2000). However, when attempting to evaluate the physiological effects of short-term psychological stressors the effectiveness of such techniques is diminished. Instead, assessment of physiological characteristics including respiration rate and skin conductance (Oetting, 1966; Sher et al., 2007) in addition to changes in heart rate and variability, blood pressure and body temperature are often utilised (Brown et al., 2006, 2003; DiDomenico and Nussbaum, 2011; Hodgson et al., 2004; Moon and Cho, 2001). Although these methods return rapid results, all are subject to considerable biological variation, introducing uncertainty during comparison between individuals and populations.

The Leukocyte Coping Capacity (LCC) test monitors the multifaceted effects of stress using the body's leukocytes (primarily, but not exclusively, neutrophils) as bio-indicators (Shelton Rayner et al., 2011, 2010). These cells circulate throughout the body picking up and responding to all of the signals of stress (illustrated in Figure 1). Leukocytes (primarily, but not exclusively, neutrophils) have over 150 different receptors which can respond to a diverse range of factors, all of which are sensitive to stress (Mian et al., 2005). 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, 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. Leukocyte Coping Capacity (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 (Shelton-Rayner

et al., 2011). 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 (e.g. Phorbol 12-myristate 13-acetate). This is the underlying technical foundation of the test (Mian et al., 2005).

The mechanism for leukocyte activation is extremely complex, involving numerous biochemical cascades and receptor interactions. Although the exact mechanism of leukocyte activation has not yet been fully elucidated, evidence does exist to argue that there is no generic response, and that leukocytes respond differently, in terms of activation state and distribution pattern according to the nature, intensity and duration of the stressor (Neveu, 2003).

Evidence shows that psychological stress modifies the effectiveness of the immune system, which can lead to increased risk of infection or disease (Altemus et al., 2003; Boscarino et al., 1999; Dhabhar et al., 1997, 1996). Epidemiological evidence indicates that individuals who are psychologically stressed are more susceptible to opportunistic infection (Clover et al., 1989; Galinowski, 1997) and demonstrate increased severity of chronic disorders, such as bronchial asthma (Joachim et al., 2008). Clark (2002) and Miller et al. (2002) demonstrated that stressor duration influences the nature of the immune response, chronic stressors (lasting days to weeks), have been shown to suppress immune responsiveness. Conversely, Viswanathan et al. (2005) demonstrated how acute psychological stressors (lasting minutes to hours) induced potent immune-enhancing effects. These can be both detrimental (through exacerbation of immune-pathological disease) and beneficial, promoting immune-protection during wounding or infection (Dhabhar, 2008).

The stress response is a complex combination of metabolic, neuroendocrine and behavioural changes. Even short-term psychological stressors such as academic examinations (Kang et al., 1996; Maes et al., 1998) can produce demonstrable physiological changes in the reactivity of specific classes of leukocyte, notably neutrophils (Mian et al., 2003). Activated neutrophils release an array of mediators, including reactive oxygen species (Ruotsalainen et al., 1995). Chemiluminescent assay of Phorbol 12-myristate 13-acetate induced reactive oxygen species production

by leukocytes has been shown to provide quantitative links between psychological anxiety and immune-competency (McLaren et al., 2003; Montes et al., 2004, 2003; Shelton-Rayner et al., 2011, 2010; Shelton-Rayner, 2009). The Leukocyte Coping Capacity (LCC) technique involves measuring leukocytes' ability to produce a respiratory burst, assayed in terms of Reactive Oxygen Species and calibrated through the emission of photons via their interaction with Luminol when stimulated using Phorbol 12-myristate 13-acetate *in vitro* (McLaren et al., 2003).

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 (Dhabhar et al., 1995; Gleeson and Bishop, 2000; Maes et al., 1998; Rosenberger *et al.*, 2009). Stress has been shown to influence the number, distribution and activation state of neutrophils in the blood in a rapid and reversible manner (Dhabhar et al., 1995; Goebel and Mills, 2000; McLaren et al., 2003; Mian et al., 2003; Shelton-Rayner et al., 2011, 2010). When compared to the stressor paradigm utilised during this research, all previous examples utilised stressors that were of far greater duration and/or intensity. This study aims to explore the potential benefits of using altered innate immune responsiveness as a means of objectively assessing altered short-term psychological stress and mental workload resulting from environmental interaction in humans. The stressor paradigm we explored involved exposing volunteers to two ergonomically different unfamiliar motor vehicles.

2.0   MATERIAL AND METHODS

2.1 The Subjects

Local ethical committee approval from Coventry University Ethics Committee and informed consent was obtained from each subject before commencement of the study, in accordance with the declaration of Helsinki (World Medical Association, 2004).

The study consisted of two phases, separated by a 2 month interval to accommodate test vehicle availability. Phase 1 consisted of 21 subjects (12 male and 9 female), phase 2 consisted of 18 different subjects (8 male and 10 female). All were moderately fit and healthy, aged between 26 and 55 years. Potential subjects were excluded on the following criteria: suffering from psychiatric illness; suffering from respiratory or cardiovascular disease; smokers; had taken prescription medicine within the previous month, and if they possessed any prior knowledge or experience regarding the test equipment.

2.2 Design

The evaluation of psychological stress exhibited as a consequence of ergonomic design, as well as for stressors in general, relies heavily upon highly subjective methodologies (e.g. subjective workload assessment technique and NASA-task load index). Quantitative techniques including, measurement of changes in heart rate (Blechert et al., 2007), blood pressure (Manikonda et al., 2008; Richman et al., 2007), body temperature (Barnum et al., 2007; Bhatnager et al., 2006) and bio-mediator concentration (e.g. cortisol) (Allen, 2007; Nij et al., 2007), are influenced by a diverse array of factors other than psychological stress, resulting in inaccurate measurement. Psychological stress reduces leukocyte responsiveness to Phorbol 12-myristate 13-acetate induced reactive oxygen species production, *in vitro* (McLaren et al., 2003). Using chemiluminescence, reactive oxygen species concentration can be measured providing an objective, rapid assessment of an individual's physical response to psychological stress (Shelton-Rayner et al., 2010; Shelton-Rayner, 2009). LCC was used to assess changes in mental loading associated with completing the same basic driving related tasks using two motor vehicles from two different manufacturers. The study design aimed to investigate the psycho-physiological response of a novice user to two completely unfamiliar in-car ergonomic environments. The task of driving itself evokes high mental workload, which is vastly increased when required to use an unfamiliar motor vehicle. The chosen tasks, albeit basic, were selected to simulate the potentially hazardous scenario of a driver being given the keys to a new unfamiliar vehicle and being immediately permitted to drive on public roads without the requirement for initial systems orientation and tuition.

The experimental protocols were rigorously standardised, and testing occurred between two time slots (11.00-12.00 hours and 14.00-15.00 hours). Subjects were required to avoid any strenuous activity for at least 2 hours prior to testing (e.g. they were instructed to take the lift to the laboratory, rather than climb the stairs). Perceived psychological stress was established pre- and immediately post-test for each stressor (Car A and B) by means of Likert scales (using a scale with 1 representing relaxed and 10 stressed) (Gaither et al., 2008; Hassinger et al., 1999). Subjects then sat quietly and were instructed to breathe orthonasally for 15 minutes prior to obtaining resting heart rate, blood pressure and core body temperature, following the standardised procedure outlined below and illustrated in Figure 2. The first pair of capillary blood samples were then taken 45 minutes before exposure to the test apparatus (45 minutes pre-stressor) (see below).

During the 45 minute Pre-Stressor period subjects were taken from the laboratory (Coventry University) to Jaguar Cars Ltd. research and development centre at Whitley, Coventry, United Kingdom – a 5 minute car journey. In an attempt to minimise the possibility that the events associated with subject transport influenced leukocyte reactivity, upon arrival, subjects were instructed to sit quietly in the researcher's vehicle for 10 minutes.

Two minutes prior to entering the test vehicle (Phase 1 – Car A, Phase 2 – Car B) the examiner explained the test protocol. The test lasted a maximum of 20 minutes. Upon successful entry into the test vehicle the subject was to adjust correctly the driving environment so as to meet British Motoring Standards, this was achieved by making the following adjustments.

1. Driver's seat (including distance from pedals, angle of back rest, seat height)
2. Position of steering wheel (rake and reach)
3. Near and offside wing mirrors
4. Rear view mirror
5. The subject was required then to start the vehicle, select the correct gear and release the handbrake. Then perform a simple manoeuvre involving moving

the car out of a pre-determined car parking space and travelling 40 metres along an access road at 3 mph, stop, select the correct gear and reverse back down the road re-entering the original parking space.

6. Finally, safely stop the engine and hand the examiner the key.

It was explained that once the test had begun no further verbal communication was permitted, and that assistance would only be provided after 3 minutes of attempting to complete the task, followed by the next instruction. Immediately upon completion of the task (Immediately post-stressor), heart rate, blood pressure and core body temperature, and perceived stress level (Likert scale) were recorded and further blood samples taken (Figure 2).

2.3 Heart Rate, Blood Pressure and Core Body Temperature Measurements

At each specified time point (Figure 2) a heart rate transceiver (Polar 610i™ Heart rate monitor, Polar Electro, Finland) attached directly to the chest, using the belt provided, monitored heart rate. Systemic blood pressure was measured using an oscillometric wrist mounted blood pressure monitor (Omron RX-3, Omron Healthcare Inc. Illinois, 60015. U.S.A.). Core body temperature was measured using an infra-red ear thermometer (Braun® Thermoscan™, P and G Brooklands, Waybridge, AT13 0XP. United Kingdom).

2.4 Blood Samples

At each specified time point (Figure 2) two 10µl blood samples were taken using a finger lancing device (Accu-Chek® Softclix®, Roche® Ltd, East Sussex, United Kingdom) from the subject's non-contractual hand (Figure 3). Following the procedure illustrated in Figure 4, one sample was used for the (non-stimulated) control (Sample A) and was placed into 10µl of murine heparin (concentration 0.1units) (CP Pharmaceuticals Ltd, Ash Road North, Wrexham, LL13 9UF, United Kingdom), 10µl Phosphate Buffered Saline (Sigma Aldrich, Dorset, SP8 4XT, United Kingdom) and 90µl of 10^{-4}M Luminol ($C_8H_7N_3O_2$) (Sigma Aldrich, Dorset, SP8

4XT, United Kingdom). The second blood sample (Sample B) was added to the same reagents, except that the 10µl of phosphate buffered saline was replaced by 10µl of 10^{-5}M Phorbol 12-myristate 13-acetate (Sigma Aldrich, Dorset, SP8 4XT, United Kingdom). Phorbol 12-myristate 13-acetate 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) (Dahlgren, 1987).

2.5 Determining Leukocyte Activity

Leukocyte Coping Capacity (LCC) is a measure, made using a Luminometer, of the concentration of Reactive Oxygen Species 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 (McLaren et al., 2003). LCC is defined as the response of leukocytes (mainly neutrophils) to challenge, in this case by Phorbol 12-myristate 13-acetate. 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).

In this experiment, each pair of blood solutions (sample A and sample B) was simultaneously tested every 5 minutes using a Luminometer (Berthold® Technologies, Junior™ LB9509, Hertfordshire, AL3 7LZ United Kingdom) for a total of 45 minutes (Figure 3), in order to produce a luminescence profile (Figure 5). Between chemiluminescence measurements the samples were incubated at 37°C in a water bath (JB1™ Grant Instruments, Cambridge United Kingdom). At each 5 minute 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 Phorbol 12-myristate 13-acetate stimulation) from the Phorbol 12-myristate 13-acetate challenge sample (Sample B).

2.6 Data Analysis

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For all measured parameters data are expressed as mean post-stressor changes ± standard error of mean (S.E.M.). For T-max (time taken to reach maximum leukocyte activity), the data were classed as discontinuous as leukocyte activity was measured at 5 minute intervals for a total of 45 minutes, in this case the median ± S.E.M. is presented. Single factor analysis of variance (ANOVA) (Statistical Package for Social Science statistical software (release 15.0) Lead Technologies Inc.) was used to test in turn, the effect of experimental group (Car A n=21 subjects and Car B n=18 subjects) on leukocyte activity, heart rate, blood pressure, core body temperature, and perceived psychological stress rating (Likert scale). ANOVA was also used to compare in turn, the effect of experimental group (Car A n=21 subjects and Car B n=18 subjects) on pre- and post-stressor leukocyte activity (using in turn, maximum adjusted leukocyte activity (Hmax-RLU$_{adj}$) and adjusted activity at 5, 10 and 15 minutes into the 45 minute luminescence profile) in addition to each of the other assessed physiological and psychological parameters (heart rate, blood pressure, core body temperature and perceived stress rating).

3.0 RESULTS

3.1 Leukocyte Activity

LCC profiles are displayed on Figure 5a and b, with post-test changes in activity for the 5 attributes of the luminescence profiles given in Figure 6. Data are expressed as mean differences between leukocyte activity 45 minutes pre-and immediately post-stressor (RLU$_{adj}$ ± standard error of mean (S.E.M.) for all parameters except T-max where median values ± S.E.M. are presented). Pre-stressor activity for both treatment groups followed the same trend with regards to both the rate and magnitude of reactive oxygen species release (Figure 5). Following the test, the mean LCC response for both treatment groups showed decreases in leukocyte activity, with the most pronounced post-stressor change occurring at T=15 minutes (RLU$_{adj}$) (adjusted response in leukocyte activity recorded 15 minutes into the 45 minute activity profile) for Car A (Car A 1198.6 ± 478.3 RLU$_{adj}$; Car B 511.5 ± 150.6 RLU$_{adj}$). These differences were found to be significant between treatment groups for T=5 minutes

(RLU$_{adj}$) (F$_{1,38}$ = 5.94, P = 0.02), T=10 minutes (RLU$_{adj}$) (F$_{1,38}$ = 4.35, P = 0.04), and for T-max (minutes) (the time taken to reach maximum adjusted leukocyte activity (Hmax-RLU$_{adj}$) (F$_{1,38}$ = 7.86, P = 0.008), (Figure 6).

3.2 Perceived Stress

Trends in post-stressor qualitative perceived stress (assessed using Likert scales) paralleled those in LCC scores, with subjects exhibiting a significant post-stressor increase of 2.0 ± 1.0 Units (F$_{1,41}$ = 7.61, P = 0.009) in response to Car A, whilst showing no significant post-stressor change in response to Car B (0.0 ± 0.0 Units (F$_{1,35}$ = 0.009, P = 0.93). As with leukocyte activity, post-stressor differences were significant between treatment groups (F$_{1,77}$ = 4.0, P = 0.05).

3.3 Core Body Temperature

No significant difference was shown between post-stressor increases in core body temperature between treatment groups (F$_{1,38}$ = 0.54, P = 0.47). The response of subjects to Car A showed a significant increase between 45 minutes pre- and immediately post-stressor of 0.4 ± 0.1°C (F$_{1,38}$ = 4.46, P = 0.04). Whilst subjects exposed to Car B showed no significant change (0.3 ± 0.1°C (F$_{1,38}$ = 86, P = 0.29).

3.4 Heart Rate, Blood Pressure

There were no significant post-stressor differences in either heart rate or systolic or diastolic blood pressure between 45 minutes pre- and immediately post-stressor, or between treatment groups.

4.0   DISCUSSION AND CONCLUSION

The results reveal that exposure to mild short-term psychological stress evokes rapid and reversible changes in immune responsiveness, changes that permit the quantitative differentiation between two closely related psychological stressors. The post-stressor change in Phorbol 12-myristate 13-acetate induced leukocyte activity was significantly greater for Car A compared to Car B (Figure 5a and b, and Figure 6). Indicating the ability of leukocytes to produce a respiratory burst in response to *in vitro* Phorbol 12-myristate 13-acetate stimulation, following completion of the stressor protocol using Car A, was significantly reduced compared to the use of Car B. As qualitative data showed that subjects perceived completion of the protocol using Car A more psychologically stressful, the degree of physiological stimulation, through hypothalamic-pituitary-adrenal axis activity (Beuschlein et al., 2001; Cohen et al., 2003) resulted in a greater *in vivo* respiratory burst for treatment group A, leading to the observed reduced *in vitro* Phorbol 12-myristate 13-acetate stimulated respiratory burst.

Ideally a within subject design for the main predictor of interest, where each subject is measured under each level of the predictor (allowing each subject to act as their own control, thus eliminating among subject variation), is preferable over the between subjects design used in this study. Unfortunately due to test vehicle availability the study had to be conducted in 2 different phases with a 2 month interval between (Phase 1 Car A n=21 and Phase 2 Car B n=18). Ideally both vehicles should have been available at the same time, and a randomised crossover design employed. However, as this was not possible it was decided to use two different volunteer groups. In not allowing any of the volunteers from Car A group to participate in the Car B group the aim was to reduce the possibility of prior knowledge of the protocol which could have potentially influenced the results. Unfortunately, it was possible to only recruit 18 subjects who met the selection criteria (described in section 2.1) for Car B group, whereas 21 different subjects had been previously selected and participated in Car A group. However, with non trivial sample sizes the between subjects design is perfectly robust, and there is no evidence that the subjects were recruited with any systematic bias regarding the treatment groups.

The Likert Scale is a well documented technique that has been used to assess perceived mental workload (Gaither et al., 2008, Hassinger et al., 1999). In our study Car A was perceived to have a higher associated mental workload (more stressful) than Car B. Trends in post-stressor qualitative perceived stress (assessed using Likert scales) paralleled those in LCC scores.

This study had sufficient power to demonstrate significant changes in 21 subjects. Stress has a wide and varied effect on humans (Mian et al., 2005). What we have demonstrated in this study is that in otherwise healthy individuals short-term exposure to psychological stress produces qualitative and quantitative changes that can easily be detected from a single drop of blood. The effects are significant because LCC monitors the multifaceted effects of stress. Other studies, for example those that use cortisol, only monitor a single aspect of stress. The variability in monitoring one parameter is much greater than monitoring the multifaceted effects of stress as measured by the LCC technique (Dokoumetzidis et al., 2002). The LCC technique mitigates differences in responsiveness to stress by monitoring the multifaceted effects of stress (Figure 1).

McLaren et al. (2003) and Naccache et al. (1990) both stated that the maximum rate of superoxide production is reached 15 minutes after Phorbol 12-myristate 13-acetate introduction, the reaction would then plateau before gradually decreasing to baseline activity. Consistent with these findings, this experiment showed a peak output between 15 and 20 minutes, with the rate of increase and magnitude of response decreasing according to psychological stressor intensity.

Interestingly, no significant changes in heart rate, systolic or diastolic blood pressure were noted between treatment groups. Numerous studies have demonstrated the ability of these traditional parameters to respond, in a rapid and reversible manner, to the presence of a psychological stressor. Blechart et al. (2007) showed how an underlying chronic stressor such as post traumatic stress disorder significantly influenced heart rate during acute psychological stress, resulting in a blunted response, when compared to healthy controls. While, Manikonda et al. (2008) demonstrated how contemplative meditation reduced ambulatory blood pressure and

stress induced hypertension. Although the duration of exposure in these examples was similar, the magnitude of the stressor paradigm utilised in this study was simply too subtle to elicit a significant change in heart rate and blood pressure.

As leukocyte activity was the only parameter demonstrating significant changes in the difference between pre- and post-stressor activity and in the magnitude of response between treatment groups, following stressor exposure. It is suggested that the stress threshold, required for leukocyte activation is lower than that needed for the other stated parameters. Leukocytes are 3 dimensional entities. Their ability to produce reactive oxygen species is altered by cell signalling pathways of other entities and cells (Shelton-Rayner et al., 2010).

The LCC test monitors the cellular capacity of leukocytes to produce superoxide radicals in real time. By deliberately leaving the cells in contact with the circulating mediators of stress, present within blood, the leukocytes are able to actively interact with other cellular components and mediators released as a consequence of stressor exposure. Leukocytes (primarily neutrophils) have over 150 different receptors which can respond to a diverse range of factors, all of which are sensitive to stress (Mian et al., 2005). Since leukocytes remain suspended in whole blood throughout the protocol, cellular integrity is maintained and potential disruption to cell signalling pathways is minimised. The process also permits 3 dimensional exposure to blood allowing the cells to dynamically interact with hormones (which can alter reactivity of the cells), other cells such as macrophages, other neutrophils, the haematocrit, and erythrocytes (whose viscosity alter during stress) (Mian et al., 2005; Shelton-Rayner et al., 2011, 2010).

During LCC analysis, leukocytes (mainly neutrophils) are stimulated *in vitro* with Phorbol 12-myristate 13-acetate and their superoxide producing capacity is measured in real time. As leukocytes release reactive oxygen species in response to stress (McLaren et al., 2003), the stimulation allows us to evaluate the leukocyte's (predominantly, but not exclusively neutrophils) capacity to generate further reactive oxygen species (Shelton-Rayner et al., 2011). 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.

Traditionally the automotive industry has used verbal feedback, Likert scales and basic physiological assessment (changes in heart rate, blood pressure and body temperature) as a means of assessing ergonomic design. The subjective nature of verbal feedback and Likert scales, and the fact that heart rate, blood pressure and body temperature responses differ between individuals and also according to the physiology of the individual at the exact time of stressor exposure, means that these parameters can only provide a basic, reactive indication of altered stress level. In contrast Phorbol 12-myristate 13-acetate stimulated leukocyte reactive oxygen species production has the potential to offer an objective means of quantifying alterations in stress level for an individual that permits direct comparison (the LCC technique is adjusted for individual baseline leukocyte activity) with the wider population.

The potential industrial applications for the LCC technique are numerous and diverse, possible uses could include designing equipment, systems and working environments to optimise their use for human capabilities and limitations. New applications could involve computers, process controls, transport and communications. Any system involving interaction with humans could be optimised using this technique. Minor and major stressors can be objectively minimised to maximise design capabilities. This could be particularly important in high-hazard and safety-critical systems and for any system where operator stress may influence the outcome of the process.

The ability to discriminate objectively between similar short-term stressors, where traditional measures were unable to identify any significant difference, underlines the importance of *in vitro* Phorbol 12-myristate 13-acetate induced leukocyte reactivity as a highly sensitive quantitative measure of mental workload. LCC analysis may also prove a useful tool in objectively quantifying stress occurring as a result of particularly subtle and transient changes in workload.

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(Taken from Shelton-Rayner et al., 2010)

Flow diagram showing factors believed to affect the activation state of leukocytes.

FIGURE 2   Sampling Protocol

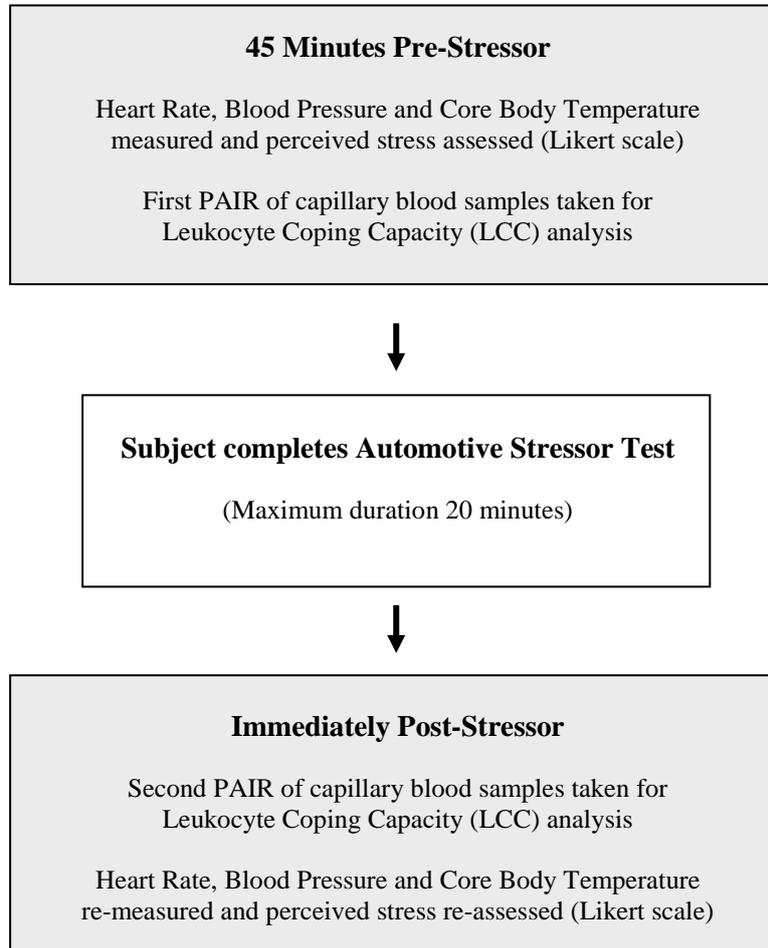(Adapted from Shelton-Rayner et al., 2010)

45 Minutes Pre-Stressor

Heart Rate, Blood Pressure and Core Body Temperature
measured and perceived stress assessed (Likert scale)

First PAIR of capillary blood samples taken for
Leukocyte Coping Capacity (LCC) analysis

↓

Subject completes Automotive Stressor Test

(Maximum duration 20 minutes)

↓

Immediately Post-Stressor

Second PAIR of capillary blood samples taken for
Leukocyte Coping Capacity (LCC) analysis

Heart Rate, Blood Pressure and Core Body Temperature
re-measured and perceived stress re-assessed (Likert scale)

Flow diagram illustrating the time line for blood sample collection, for Leukocyte
Coping Capacity (LCC) analysis, and measurement of other physiological (Heart
Rate, Systolic and Diastolic Blood Pressure, and Core Body Temperature) and
psychological (Perceived Stress Rating) parameters.

FIGURE 3 Key stages in the Leukocyte Coping Capacity (LCC) protocol

STAGE 1

Using a finger lancing device (Accu-Chek® Softclix®, Roche® Ltd) puncture skin of a finger on the subject's non-contractual hand.
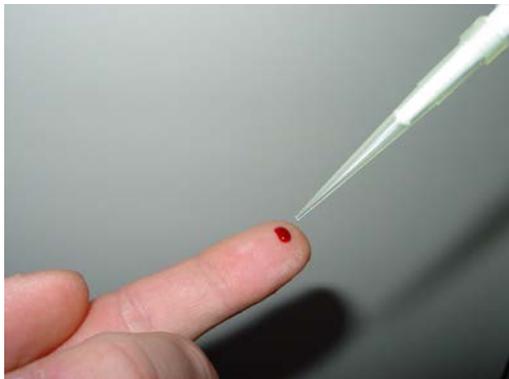
STAGE 2

Using a P10 pipette obtain <u>two</u> 10µl Capillary Blood Samples
(Sample A and Sample B)

STAGE 3

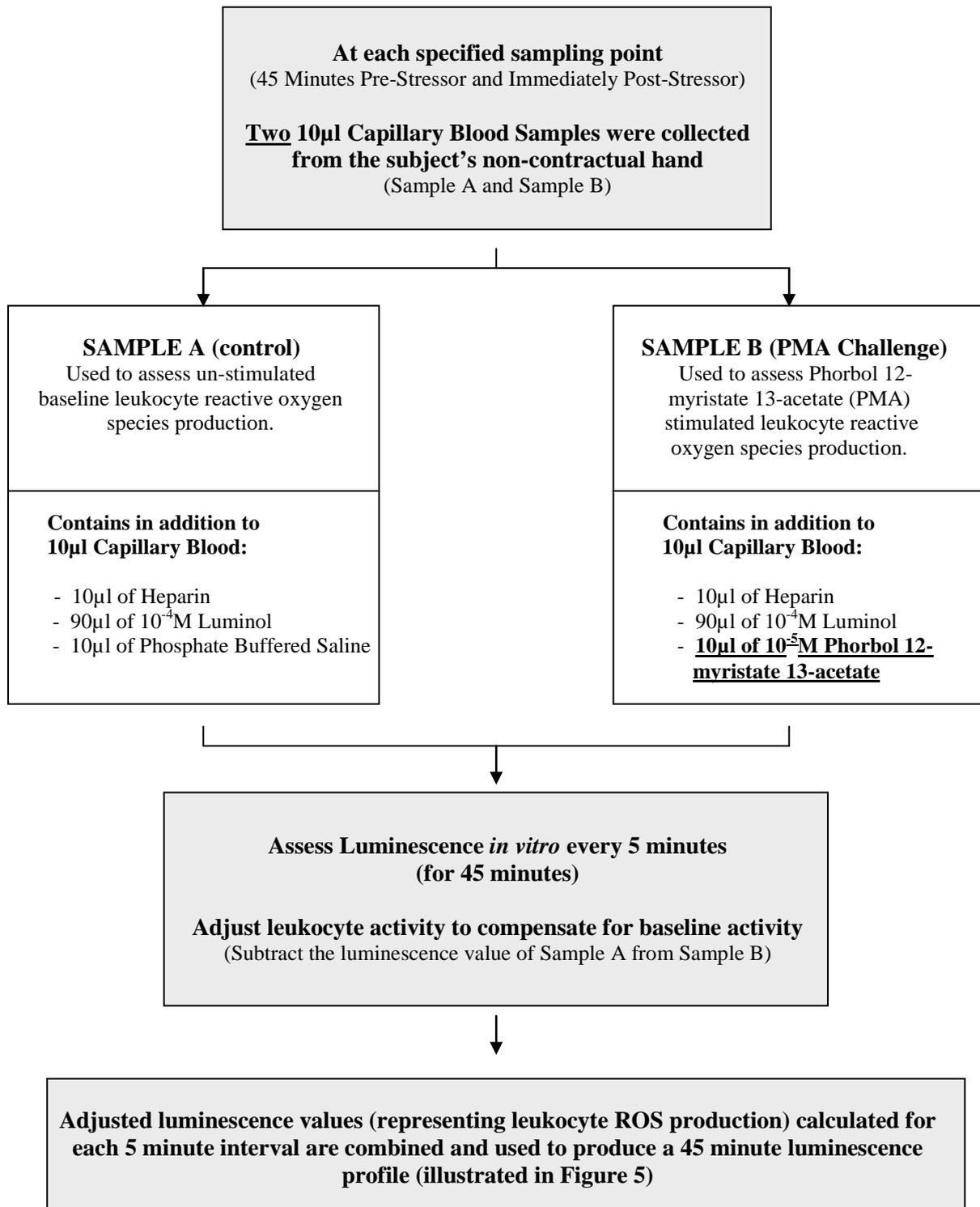Add one of the 10µl Capillary Blood Samples to each of the pre-prepared reagent solutions (Sample A – Control and Sample B – Phorbol 12-myristate 13-acetate Challenge) (Reagent formula described in Figure 4), mix and immediately assess luminescence using a luminometer (Berthold® Technologies, Junior™ LB9509). Measure luminescence of each solution at 5 minute intervals for 45 minutes; incubate at 37°C between measurements.

FIGURE 4   Leukocyte Coping Capacity (LCC) Protocol

(Adapted from Shelton-Rayner et al., 2010)

At each specified sampling point
(45 Minutes Pre-Stressor and Immediately Post-Stressor)

**<u>Two</u> 10µl Capillary Blood Samples were collected
from the subject's non-contractual hand**
(Sample A and Sample B)

SAMPLE A (control)
Used to assess un-stimulated baseline leukocyte reactive oxygen species production.

**Contains in addition to
10µl Capillary Blood:**

- 10µl of Heparin
- 90µl of 10^{-4}M Luminol
- 10µl of Phosphate Buffered Saline

SAMPLE B (PMA Challenge)
Used to assess Phorbol 12-myristate 13-acetate (PMA) stimulated leukocyte reactive oxygen species production.

**Contains in addition to
10µl Capillary Blood:**

- 10µl of Heparin
- 90µl of 10^{-4}M Luminol
- **<u>10µl of 10^{-5}M Phorbol 12-myristate 13-acetate</u>**

**Assess Luminescence *in vitro* every 5 minutes
(for 45 minutes)**

Adjust leukocyte activity to compensate for baseline activity
(Subtract the luminescence value of Sample A from Sample B)

Adjusted luminescence values (representing leukocyte ROS production) calculated for each 5 minute interval are combined and used to produce a 45 minute luminescence profile (illustrated in Figure 5)

Leukocyte activity was assessed using whole blood samples taken 45 minutes pre and immediately post-stressor, following the protocol illustrated.
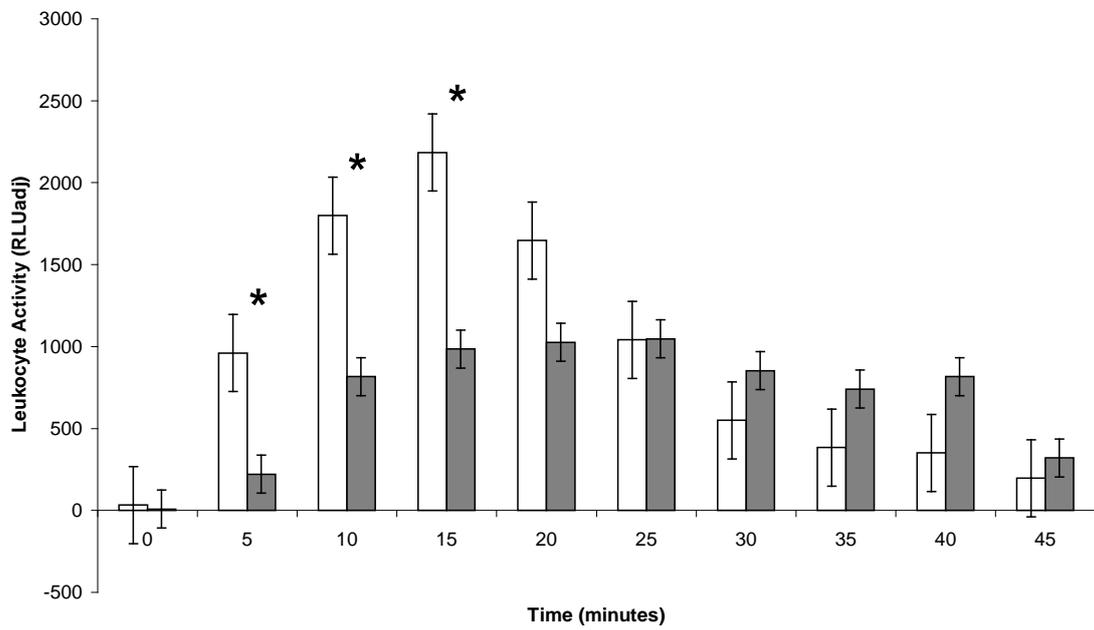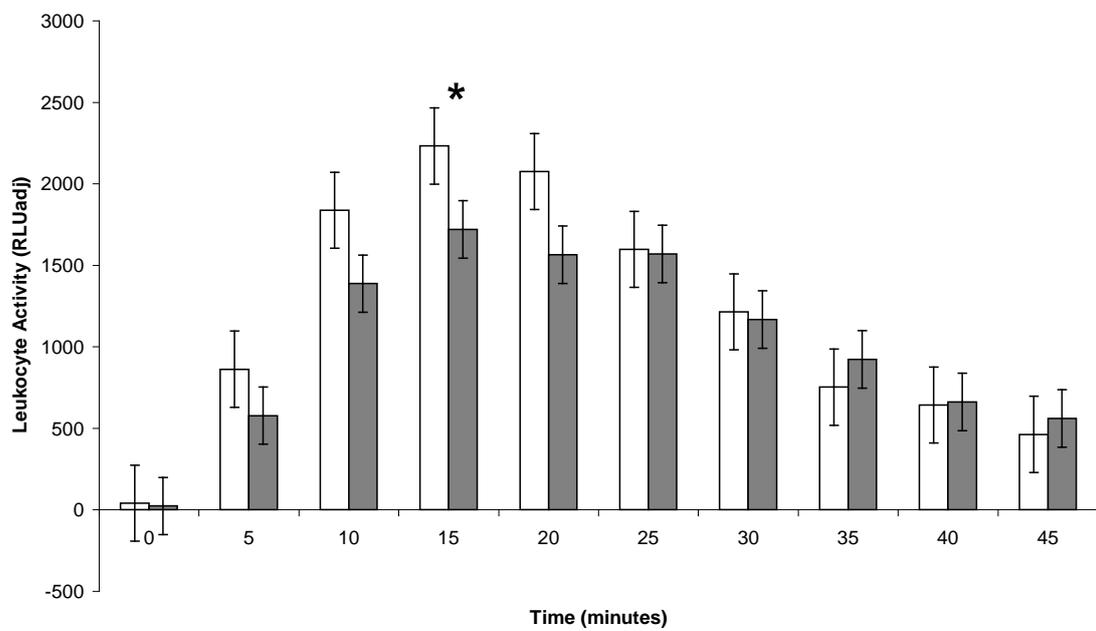
FIGURE 5a   Treatment Group A (Car A) (n=21)

FIGURE 5b   Treatment Group B (Car B) (n=18)

Mean adjusted Leukocyte Coping Capacity (RLU$_{adj}$) ± standard error of the mean (S.E.M.) for treatment groups A (n=21) and B (n=18). White bars represent mean adjusted leukocyte activity 45 minutes pre-stressor and Black bars represent activity immediately post-stressor. * indicates significant difference in activity between 45 minutes pre-and immediately post-stressor ($P < 0.05$).

FIGURE 6   Effect of stressor on leukocyte activity
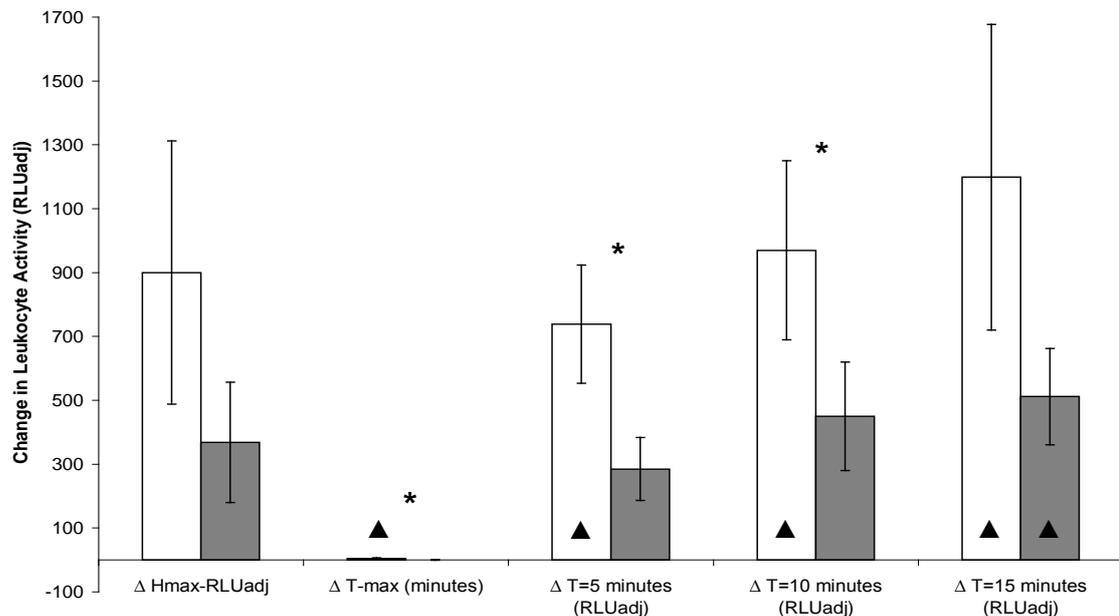
Mean and standard error of the mean (S.E.M.) are presented for the change (Δ) in leukocyte activity (difference between 45 minutes pre- and immediately post-stressor samples) for four specific attributes of the leukocyte luminescence profiles for treatment group A (Car A) (n = 21) and treatment group B (Car B) (n = 18) (Leukocyte Activity - Adjusted Relative Light Units – RLU_{adj}), White bars represent Car A and Black bars Car B. Hmax (RLU_{adj}) - the maximum adjusted response exhibited during the 45 minute sampling period, T=5, 10 and 15 minutes - the adjusted response in leukocyte activity recorded at 5, 10 and 15 minutes into the 45 minute activity profile (RLU_{adj}). T-max data (difference in the time taken to reach maximum adjusted leukocyte activity between 45 minutes pre- and immediately post-stressor) is discontinuous; therefore median values with S.E.M are presented. Single factor ANOVA was used to investigate the effect of treatment on leukocyte activity (*d.f.* = 38).

▲ Difference between Pre- and Post-Stressor Leukocyte Activity (*P* < 0.05)

* Difference between Treatment group A (Car A) and Treatment group B (Car B) (*P* < 0.05)