Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals and consumables

All chemicals used in the experimental work presented in this thesis were of analytical grade or higher and were obtained from the manufacturers or suppliers listed below.

Ajax Chemicals (Auburn, Sydney, NSW): Potassium dihydrogen orthophosphate.

Amersham-Pharmacia (Baulkham Hills, Sydney, NSW): Cellophane sheets, Enhanced Chemilluminescence (ECL™) western blotting detection kit, Ficoll-Paque® plus, nitrocellulose membrane (Hybond-C super), Hyperfilm™-ECL™.

Astral Scientific (Gymea, Sydney, NSW): Freezing storage containers.

Becton Dickinson Biosciences (North Ryde, Sydney, NSW): BD Vacutainer 6 ml sodium heparin (NH 102 IU) blood collection tubes, BD Vacutainer 10 ml lithium heparin blood collection tubes, BD FACS lysing solution, BD FACS permeabilizing Solution 2, Falcon 5 ml polystyrene round bottom flow cytometry tubes with lids.

BHD/Merck Chemicals (Kilsyth, Melbourne, Victoria): Acetic acid, acrylamide, bromophenol blue, disodium hydrogen orthophosphate, ethanol, glycerol, hydrochloric acid, isopropanol, magnesium chloride, N,N,'-methylenebisacrylamide, potassium chloride, sodium chloride, sodium dihydrogen orthophosphate, sodium hydroxide, N,N,N,N'-tetramethylenediamine (TEMED), trichloroacetic acid (TCA).

Bio-Rad (Regents Park, Sydney, NSW): Cellophane membrane backing, filter backing paper, Kaleidoscope pre-stained standards, pre-stained SDS-PAGE standards (broad range), SDS-PAGE molecular weight standards (low range).

Boehringer Mannheim (Castle Hill, Sydney, NSW): Phenylmethylsulphonyl fluoride (PMSF).


Crown Scientific (Moorebank, Sydney, NSW): IWAKI sterile 50 ml centrifuge tubes, Novix II parafilm.

CSL Biosciences (Parkville, Melbourne, Victoria): Foetal bovine serum (FBS).
Chapter 2: Materials & Methods

CSR (Pyrmont, Sydney, Australia): Absolute ethyl alcohol (99.8%), methyl alcohol.
Diploma (Melbourne, Victoria): Skim milk powder.
Gibco-BRL (Mulgrave, Melbourne, Victoria): RPMI medium 1640 w/o methionine.
Gradipore Ltd. (Frenchs Forest, Sydney, NSW): 10% Pre-cast polyacrylamide electrophoresis gels (iGels).
Greiner Bio-One GmbH (Kremsmuenster, Austria): 9 ml lithium heparin Vacuette® blood collection tubes.
ICN Biomedicals (Seven Hills, Sydney, NSW): Acrylamide (electrophoresis grade), ammonium peroxodisulphate (AMPS), 2,4'-dinitrophenyl hydrazine (DNPH), ethyl acetate, glycine, guanidine hydrochloride, sodium dodecyl sulphate (SDS), trizma base (Tris).
ICN Biomedicals Radiochemicals Division (Irvine, California, USA): Trans $^{35}$S-Label™ ($^{35}$S] labeling reagent, containing 70% L- Methionine, [$^{35}$S], and 15% L-Cysteine [$^{35}$S]).
Indoplas (Mona Vale, Sydney, NSW): sterile mixing canulae
JRH Biosciences (Brooklyn, Melbourne, Victoria): PBS-Dulbecco’s PBS (DPBS modified) w/o calcium, w/o magnesium.
Livingstone International Pty. Ltd. (Rosebery, NSW): Latex examination gloves.
Pierce (Laboratory Supply, Marrickville, Sydney, NSW): Coomassie protein assay reagent, albumin standard (fraction V, 2 mg/ml).
ProSciTech (Kirwan, Qld): Formaldehyde EM grade solution 16%.
Sarstedt (Ingle Farm, SA): 10 ml centrifuge tubes, 1 ml microfuge tubes, sterile transfer pipettes, sterile 10 ml and 25 ml pipettes, pipette tips, disposable cuvettes.
Sigma-Aldrich Incorporated (Castle Hill, Sydney, NSW): 2,4-dinitrophenylhydrazine (DNPH), ethyl acetate, polyoxyethylene-sorbitan monolaurate (Tween 20), 2-thiobarbituric acid, Triton X-100, albumin (Bovine), trypan blue solution (0.4%).
StressGen Biotechnologies Corporation (Victoria, British Columbia, Canada): StressXpress™ Hsp70 ELISA kit (EKS-700).
Terumo® (Tokyo, Japan): 5 ml, 10 ml and 20 ml syringes and needles.
Wako Pure Chemical Industries (Novachem, Collingwood, VIC): 2,2'-Azobis-(2-amidinopropane) dihydrochloride (AAPH).
Whatman® (Laboratory Supplies, Marrackville, NSW): Filter paper (grade 41, 150 mm diameter).
2.1.2 Antibodies

2.1.2.1 Anti-hsp antibodies

Sigma-Aldrich Incorporated (Castle Hill, Sydney, NSW): Mouse monoclonal anti-β-actin (mouse IgG1 isotype) (A5441).

StressGen Biotechnologies Corporation (Victoria, British Columbia, Canada): Mouse monoclonal anti-human hsp70 antibody (SPA-810), rat monoclonal anti-human hsp90α antibody (SPA-840).

2.1.2.2 Secondary antibodies

Amersham-Pharmacia (Baulkham Hills, Sydney, NSW): Sheep anti-mouse IgG conjugated to horseradish peroxidase was supplied in the ECL™ western blotting detection kit.

Rockland Immunochemicals Incorporated (Gilbertsville, Pennsylvania, USA): Goat anti-rat IgG (H & L) (612-1302).

2.1.2.3 Flow cytometry extracellular marker antibodies

Becton Dickinson Biosciences (North Ryde, Sydney, NSW): Anti-CD3 conjugated to PerCP (347344), anti-CD3 conjugated to PerCP-Cy5.5 (340949), anti-CD4 conjugated to PE-Cy7 (348789), anti-CD8 conjugated to APC-Cy7 (348793), anti-CD11c conjugated to PE (555392), anti-CD14 conjugated to PE (347497), anti-CD14 conjugated to APC (340436), anti-CD16 conjugated to PE (555407), anti-CD19 conjugated to PE (555413), anti-CD36 conjugated to PE (555455), anti-CD38 conjugated to APC (340439), anti-CD45RA conjugated to APC (550855), anti-CD45RO conjugated to PE (555493), anti-CD56 conjugated to APC (555518), anti-CD62L conjugated to PE (341012), anti-CD123 conjugated to PE (340545), anti-HLA-DR conjugated to PerCP (347364).

2.1.2.4 Flow cytometry intracellular marker antibodies

StressGen Biotechnologies Corporation (Victoria, British Columbia, Canada): Anti-hsp70 conjugated to FITC (SPA-810FI).

2.1.2.5 Flow cytometry control antibodies

Sigma-Aldrich Incorporated (Castle Hill, Sydney, NSW): Mouse IgG1 (kappa) immunoglobulin.
2.1.3 Software packages

Adobe (Systems Incorporated (USA): Photoshop V6.0 imaging software program.

Becton Dickinson Biosciences (North Ryde, Sydney, NSW): FACSDiva flow cytometry software package, Cell Quest V3.1f flow cytometry software package.

Labsystems (Finlans): Genesis 2 ELISA plate reader software package.

Minitab Incorporated (Pennsylvania, USA): Minitab 13 statistical analysis software package


SPSS Science Software (Chicago, USA): SigmaPlot 8.0 graphical software package.


2.2 Methods

2.2.1 General

All solutions were prepared with glass-distilled water which was further purified by the Millipore Milli-Q filtration system (Millipore, Lane Cove, Sydney, NSW). All solutions requiring sterilization were autoclaved at 121°C for 15 min. The pH of solutions was measured and adjusted as necessary using a pH meter (Activon model 209). Methodologies and conditions specific to individual studies comprising this thesis are included in the relevant chapters.

2.2.2 Study design

Each Chapter of this thesis involved the recruitment of a different cohort of participants as specified in the relevant Chapters. All participants were provided with an information form detailing the study procedures and each gave written informed consent and completed a questionnaire regarding general health and their use of medications, nutritional supplements, tobacco and alcohol. Copies of the information sheets, questionnaires and consent forms are located in Appendix I. All procedures were approved by the relevant Human Research Ethics Committees as detailed in the relevant Chapters of this thesis.
2.2.3 Blood collection

Peripheral blood samples were collected by venipuncture into lithium heparin vacutainers for analysis of leukocyte protein synthesis, plasma and erythrocyte studies. Blood was collected into uncoated vacutainers for studies requiring serum. All blood samples were collected by trained personnel at a number of pathology laboratories and medical clinics located in Armidale and Sydney. All blood samples were kept at room temperature and processed within 24 h of collection.

2.2.4 Isolation of lymphocytes

Approximately 20 ml of whole blood was used for the lymphocyte harvest. Lymphocytes were isolated from blood using the Ficoll-Paque® Plus (Amersham-Pharmacia, Baulkham Hills, Sydney, NSW) gradient centrifugation procedure as previously described (Bøyum, 1968). Briefly, 15 ml RPMI medium without methionine was added to 20 ml of whole blood in a 50 ml centrifuge tube, and gently mixed. Ficoll-Paque Plus (10 ml) was carefully underlaid taking care to maintain a perfect interface between blood/media and the Ficoll. Tubes were centrifuged at 400 x g for 30 min without acceleration or braking. Lymphocytes were aspirated and mixed with RPMI medium without methionine and re-centrifuged at 400 x g for 8 min with a 5 min brake. The supernatant was discarded and the pellet was resuspended in 10 ml RPMI medium without methionine. Tubes were centrifuged at 400 x g for 8 min with 5 min acceleration and 5 min braking. The supernatant was discarded and the lymphocyte fraction was resuspended in RPMI medium without methionine and supplemented with 10% foetal bovine serum.

2.2.5 Stress treatment

Heat shock

For heat shock, the lymphocytes were placed in a 42.5°C water bath for 1 hr, followed by a recovery period of 3 hr in a 37°C water bath. Control cells were maintained in the 37°C water bath for the entire 4 hr period.

2.2.6 Analysis of protein synthesis

Radioactive labeling

Prior to heat shock treatment, 150 μCi/ml of $^{35}$S-methionine was added to the lymphocyte suspension. The incorporation of $^{35}$S-methionine into protein enables examination of de novo protein synthesis (Coligan et al., 1983).
Protein extraction
Heat shock and control samples were centrifuged at 900 x g for 3 min, after which the supernatant was removed and the pellet resuspended in 800 μl RPMI to remove excess label. The suspension was centrifuged again for 3 min at 900 x g. The supernatant was removed and the pellet resuspended in 50 μl protein extraction buffer [10 μl 10% v/v Triton X-100, 100 μl 1000 mM-KCl, 8 μl 1000 mM-MgCl2·6H2O, 150 μl 1000 mM-NaCl, 20 μl 1000 mM-Tris-HCl pH 7.4, 10 μl 100 mM-PMSF, 702 μl ddH2O]. Samples were frozen overnight at -20°C. The following day, samples were thawed over ice with intermittent vortexing, then centrifuged at 900 x g for 3 min. The supernatant was transferred into fresh microfuge tubes and frozen at -80°C until the time of assay.

Protein assay
Protein concentrations were determined by the Coomassie blue assay as based upon the Bradford (1976) method, using BSA as a standard. Samples were thawed over ice with intermittent vortexing and protein sample (5 μl) added to 495 μl ddH2O and 500 μl Coomassie reagent. Optical density was read at 595 nm against a blank containing 500 μl ddH2O and 500 μl Coomassie. Concentrations were determined by interpolation from a standard curve constructed using BSA [0.625 – 80 μg/ml].

Electrophoresis
Protein extracts were combined with equal amounts of 3 x gel loading buffer [0.25 M Tris-HCl, pH 6.8; 10% (v/v) glycerol; 2% (w/v) SDS; 5% (w/v) 2-mecaptoethanol; ddH2O, 0.03 g bromophenol blue] and proteins were separated by SDS-PAGE (Laemmlli, 1970). Samples containing 10 μg protein were run against pre-stained molecular weight markers (Bio-Rad) (Table 2.1). Separating gels consisted of 10% (w/v) polyacrylamide [13.32 ml of 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide, 10 ml 1.5 M-Tris-HCl pH 8.8, 16.10 ml H2O, 400 μl 10% (w/v) SDS, 140 μl 10% (w/v) AMPS, 40 μl TEMED] with a 5 cm, 4% (w/v) polyacrylamide stacking gel [1.30 ml of 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide, 1.25 ml 1 M-Tris-HCl pH 6.8, 7.24 ml H2O, 100 μl 10% (w/v) SDS, 100 μl 10% (w/v) AMPS, 10 μl TEMED]. The gel running buffer contained 1 g SDS, 6 g Tris and 18.8 g glycine per litre. Electrophoresis was carried out at 10°C with a current of 30 mA, for a duration of between 5 and 6 hr. Electrophoresis was discontinued once the bromophenol blue loading dye had migrated to the end of the gel.
Table 2.1: Calibrated Molecular Weights of Prestained SDS-PAGE Standards

<table>
<thead>
<tr>
<th>Protein</th>
<th>High range Control #85053</th>
<th>Low Range Control #85440</th>
<th>Broad Range Control #85059</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin</td>
<td>204,000</td>
<td>-</td>
<td>209,000</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>123,000</td>
<td>124,000</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>-</td>
<td>103,000</td>
<td>-</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>80,000</td>
<td>50,000</td>
<td>49,100</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>48,000</td>
<td>34,300</td>
<td>34,800</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>28,800</td>
<td>28,900</td>
<td></td>
</tr>
<tr>
<td>Aprotinin</td>
<td>20,700</td>
<td>20,600</td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>-</td>
<td>-</td>
<td>7,100</td>
</tr>
</tbody>
</table>

Autoradiography

After electrophoresis, gels were fixed in a solution of 40% (w/v) methanol and 10% (w/v) acetic acid for 30 min. Gels were then dried between cellophane at 72°C for 1 hr and 10 min using a Model 583 Gel Dryer (BioRad). Dried gels were exposed to Biomax MR film (Kodak) in a cassette for 10 – 14 days at -70°C. Autoradiograms were developed by immersion in Kodak liquid X-Ray developer for 30 sec, rinsed in water, immersed in Kodak liquid fixer for 30 sec, then rinsed in water again.

Densitometry

Autoradiograms were analyzed using a gel documentation and analysis system (Ultra-Violet Products, Cambridge, UK) and the Phoretix 1D densitometry software package V2003 (Phoretix International, Newcastle, UK). Actin (43kDa), a non-stress inducible protein, was used as an internal control (Gross & Watson, 1998). The ratio of respective band intensities relative to that of actin in corresponding control and heat shock lanes was calculated and used to quantitate the particular stress proteins of interest to this study. The relative increase in protein synthesis was expressed as a ratio of normalized pixel intensity with respect to actin.
Chapter 2: Materials & Methods

2.2.7 Western Blotting Analysis

Electrophoresis
Protein samples (10-20 μg) and molecular weight standards (15 μl) were separated by 1D-SDS-PAGE (as described in Section 2.2.6) on 10% iGels (Gradipore).

Transfer of protein to membranes
Following electrophoresis, gels were immersed in continuous transfer buffer [39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS, 20% methanol] for 5 minutes. Proteins were transferred onto Hybond C super nitrocellulose membrane (Amersham) that had been pre-equilibrated in transfer buffer. The transfer was performed using a 2117-250 Novablot Electrophoresis Transfer Kit (AMRAD Pharmacia) on a stack of nine filter papers pre-equilibrated in transfer buffer, the nitrocellulose membrane, the gel and another nine filter papers soaked in transfer buffer. Transfer was carried out at room temperature, with a constant current of 120 mA for 90 min. The membrane was blocked overnight in a cool room in a blocking solution [80 mM-Na₂HPO₄, 20 mM-NaH₂PO₄, 100 mM-NaCl, 0.1% (w/v) Tween, pH 7.4 with 5% (w/v) skim milk powder].

Detection of bound antibody
Response to specific antibodies was detected using the ECL™ western blotting detection kit (Amersham) in accordance with the manufacturer's instructions. After non-specific binding sites were blocked overnight, membranes were rinsed twice in PBS-T [PBS, 0.1% (w/v) Tween-20], followed by 15 min in PBS-T and 2 x 5 min in PBS-T, all with gentle rocking. Membranes were incubated with the appropriate antibody in PBS-T with 5% (w/v) skim milk powder in a sealed plastic bag, for 1 hr at room temperature with gentle rocking. Primary antibodies were used at the concentrations listed in Table 2.2. The membranes were rinsed twice in PBS-T [PBS, 0.1% (w/v) Tween-20], followed by 15 min in PBS-T and 2 x 5 min in PBS-T, all with gentle rocking. After washing, the membranes were incubated with a secondary antibody (anti-mouse) at a concentration of 1:1000 in PBS-T with 5% (w/v) skim milk powder in a sealed plastic bag, for 1 h at room temperature with gentle rocking. The membranes were rinsed twice in PBS-T [PBS, 0.1% (w/v) Tween-20], followed by 15 min in PBS-T and 2 x 5 min in PBS-T, all with gentle rocking. The membranes were incubated with ECL™ detection reagents for 1 min, drained, then wrapped in cling-wrap and exposed to ECL™ Hyperfilm film (Amersham) for periods of between 30
sec and 3 min. Films were developed by immersion in Kodak liquid X-Ray developer for 30 sec, rinsed in water, followed by immersion in Kodak liquid X-Ray fixer for 30 sec and finally rinsed in water.

**Table 2.2: Primary antibody dilutions**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-β-actin</td>
<td>1:3000</td>
</tr>
<tr>
<td>Anti-hsp70</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-hsp90</td>
<td>1:2000</td>
</tr>
</tbody>
</table>

**Stripping of membranes**

Prior to reprobing, Western immunoblot membranes were stripped using a previously described method (Liao *et al.*, 2000). Membranes were incubated in 100 ml stripping buffer [100 mM-2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM-Tris-HCl pH 6.7] at 50°C for 30 min with occasional agitation. Membranes were washed twice in 100 ml PBS-T [PBS, 0.1% (w/v) Tween-20] for 10 min with gentle rocking at room temperature. Membranes were then blocked for 1 hr at room temperature in blocking solution [80 mM-Na₂HPO₄, 20 mM-NaH₂PO₄, 100 mM-NaCl, 0.1% (w/v) Tween-20, pH 7.4 with 5% (w/v) skim milk powder].

**Densitometry**

Western immunoblots were analyzed using a gel documentation and analysis system (Ultra-Violet Products, Cambridge, UK) and the Phoretix 1D densitometry software package V3 (Phoretix International, Newcastle, UK). The ratio of respective band intensities relative to that of actin in corresponding control and heat shock lanes was calculated and used to quanitate the particular stress proteins of interest to this study. The relative increase in protein synthesis was expressed as a ratio of normalized pixel intensity with respect to β-actin.

**2.2.8 Cryopreservation of peripheral blood mononuclear cells**

**Cryopreservation**

PBMCs were prepared by Ficoll-Paque density centrifugation then washed twice in methionine-free RPMI. Cell density was counted using the trypan blue exclusion method as detailed below. Cells were resuspended in freezing solution comprising 90% FBS and 10% (v/v) DMSO, at a concentration of between 5 x 10⁶ and 10 x 10⁶
cells/ml. Cryovial caps were securely tightened and samples were frozen overnight at -70 °C. Samples were placed in a liquid nitrogen tank for long-term storage.

**Thawing cells**
Cryovials were transferred from the liquid nitrogen tank directly to a 37 °C water bath. Cryovial lids were loosened slightly to allow any nitrogen that may have seeped inside the vial during storage to escape. Cryovials were held at the surface of the water bath and contents were gently mixed to maintain even temperature of contents. Cells were transferred to a 15 ml centrifuge tube and chilled, methionine-free complete media was added in a drop-wise manner to a volume of approximately 10 ml. Cells were washed twice with methionine-free complete media, by centrifuging at 300 x g for 10 min. Cell density and viability were determined using the trypan blue exclusion method as detailed below.

**Trypan blue exclusion**
Cell density and viability were determined using the trypan blue exclusion method as previously described (Adams, 1990). Aliquots (20 μl) of cell suspension were thoroughly mixed with an equal volume of 0.4% (w/v) trypan blue solution. The mixture was loaded into a haemocytometer, dye-negative and dye-positive cells were counted and the percentage of viable cells was determined.

**2.2.9 Hsp70 ELISA**
The StressXpress™ hsp70 ELISA kit (StressGen Biotechnologies, Victoria, Canada) was used, following the manufacturer’s instructions, to detect and quantitate levels of hsp70 in protein extracted from lymphocytes and plasma. This ELISA is a quantitative sandwich immunoassay that uses an immunoassay plate pre-coated with a mouse monoclonal antibody that is specific for inducible hsp70. The immobilized antibody captures inducible hsp70 and is detected with an hsp70 specific biotinylated rabbit polyclonal antibody that also binds with an avidin-horseradish peroxidase conjugate. The assay is developed with tetramethylbenzidine substrate and colour development is stopped with an acid stop solution. Briefly, 100 μl of hsp70 standard solutions of varying concentrations, blank solutions and samples of protein extracts (diluted 1:50 with sample diluent) or plasma samples (diluted 1:5 with sample diluent) were pipetted into a 96-well microplate that had been pre-coated with monoclonal mouse anti-hsp70. All standards, blanks and
samples were run in duplicate. The microplate was incubated for 2 h at room temperature with gentle rocking. The contents of each well were aspirated and 250 µl wash buffer was added to each well. This was then aspirated and the washing steps repeated a further five times. The microplate was patted dry with paper towel then 100 µl biotinylated rabbit anti-mouse polyclonal secondary antibody was added to each well. The microplate was covered and incubated for 1 h at room temperature with gentle rocking. The washing steps were repeated as above. The microplate was patted dry with paper towel, then 100 µl avidin-horesradish peroxidase conjugate was added to each well. The microplate was covered and incubated for 1 h at room temperature with gentle rocking. The washing steps were repeated as above. The microplate was patted dry with paper towel, then 100 µl tetramethylbenzidine substrate was added to each well. The microplate was covered and incubated for 10 min at room temperature with gentle rocking. Finally, 100 µl acid stop solution was added to each well and the colour intensity was read at 450 nm in a TiterTek Multiscan® Plus Plate Reader (Labsystems, Finland) microplate reader. A standard curve was constructed using a calibrated hsp70 protein standard and sample concentrations of hsp70 were quantitated by interpolation of absorbances from the standard curve using the Genesis 2 Software package (Labsystems, Finland).

### 2.2.10 Flow cytometric analysis of hsp70 expression in whole blood

#### General conditions

All incubation and centrifugation steps were performed at room temperature. A Sorvall 6000D centrifuge (Kendro Industries, Lane Cove, NSW) was used.

#### Stress treatment (heat shock)

For heat shock, 1 ml samples of heparinized whole blood were pre-equilibrated in a 37°C water bath for 15 min then placed in a 42.5°C water bath for 1 h, followed by a recovery period of 3 h in a 37°C water bath. Control blood samples (1 ml) were maintained in the 37°C water bath for the entire 4 h 15 min period.

#### Antibody staining, cell permeablization and fixing

Samples (100 µl) of whole blood were added to a FACS tube containing the appropriate amounts and combinations of extracellular marker antibodies (Table 2.3). The tubes were gently vortexed then incubated for 12 – 15 min. FACSLyse (2 ml) [Becton-Dickinson] was added and the tubes were vortexed gently then incubated for
10 min. The tubes were centrifuged at 1500 rpm for 7 min. The supernatant was aspirated, and 500 μl FACSPerm (Becton-Dickinson) was added. The tubes were gently vortexed and incubated for 10 min. PBA (2 ml, phosphate buffered saline + 0.5% bovine serum albumin + 0.1% sodium azide) was added to the tubes which were centrifuged at 1700 rpm for 7 min. The supernatant was carefully aspirated. Intracellular antibody (10 μl, anti-hsp70-FITC diluted 1:10 with PBA) or 20 μl control anti-IgG-FITC were added to the tubes which were vortexed gently then incubated in the dark for 30 min. PBA (2 ml) was added to the tubes, which were centrifuged at 1700 rpm for 7 min. The supernatant was carefully aspirated and 500 μl 0.5% paraformaldehyde added to the tubes. The tubes were stored at 4°C until flow cytometric analysis was performed on a BD-LSR-2 with BD-FACSDiva software. This analysis was completed within 24 h of staining.

Table 2.3: Flow cytometry extracellular marker antibodies

<table>
<thead>
<tr>
<th>TUBE A</th>
<th>μl</th>
<th>TUBE B</th>
<th>μl</th>
<th>TUBE C</th>
<th>μl</th>
<th>TUBE D</th>
<th>μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 PerCP-Cy5.5</td>
<td>20</td>
<td>CD3 PerCP-Cy5.5</td>
<td>20</td>
<td>CD3 PerCP-Cy5.5</td>
<td>20</td>
<td>CD3 PerCP</td>
<td>20</td>
</tr>
<tr>
<td>CD4 PE-Cy7</td>
<td>2</td>
<td>CD4 PE-Cy7</td>
<td>2</td>
<td>CD4 PE-Cy7</td>
<td>2</td>
<td>CD4 PE-Cy7</td>
<td>2</td>
</tr>
<tr>
<td>CD14 APC</td>
<td>5</td>
<td>CD38 APC</td>
<td>5</td>
<td>CD45RA APC</td>
<td>20</td>
<td>CD14 APC</td>
<td>5</td>
</tr>
<tr>
<td>CD16 PE</td>
<td>20</td>
<td>CD45RO PE</td>
<td>20</td>
<td>CD62L PE</td>
<td>20</td>
<td>CD16 PE</td>
<td>20</td>
</tr>
</tbody>
</table>

2.2.11 Erythrocyte haemolysis assay

Approximately 4 ml of whole blood was centrifuged at 600 x g for 10 min. Plasma was removed by aspiration and was frozen in aliquots at -80°C for later analysis. The buffy coat was removed by aspiration and discarded. Erythrocytes were washed twice with 5 volumes of phosphate-buffered saline (PBS) \([136.9 \text{ mM-NaCl, 2.68 mM-KCl, 10 mM-Na}_2\text{HPO}_4, 1.76 \text{ mM-K}_2\text{HPO}_4; pH 7.4]\) by centrifugation at 600 x g for 5 min. Erythrocyte aliquots (200 μl) were combined with 800 μl PBS and 1 ml 100 mM-AAPH in PBS, pH 7.4. The suspension was carefully mixed, and incubated at 37°C in a shaking water bath for 6 to 7 h. The optical densities of 50 μl aliquots of cell suspension in 1.95 ml 0.15 M NaCl were measured at 540 nm every 30 min. Percent haemolysis was calculated against 50 μl cell suspension in 1.95 ml ddH₂O which was taken as 100% haemolysis.
2.2.12 Total antioxidant status of plasma

Whole blood was centrifuged at 600 x g for 10 min and plasma was collected. Analysis of total antioxidant status in plasma was carried out using the Calbiochem® Total Antioxidant Status Assay Kit (Cat. No. 615700), in accordance with the manufacturer’s instructions. This assay operates on the principle that antioxidants in a sample will inhibit the oxidation of ABTS (2,2-azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS⁰⁺ by the peroxidase, metmyoglobin. Formation of ABTS⁰⁺ is monitored by reading the absorbance at 600 nm. At this absorbance, and under these reaction conditions, antioxidants in the sample will suppress the absorbance in a proportional manner to their concentration, as illustrated in Figure 2.1.

\[
\begin{align*}
HX-Fe^{3+} + H_2O_2 & \rightarrow \text{metmyoglobin} \\
*X-[Fe^{4+}=O] + ABTS & \rightarrow \text{ferrylmyoglobin} \\
+ \text{antioxidants} & \quad - \text{antioxidants} \\
\text{Reaction inhibited} & \quad \text{less ABTS}^{⁰⁺} \text{produced} \\
\text{ABTS}^{⁰⁺} + HX-Fe^{3+} & \quad (\text{Read at 600nm})
\end{align*}
\]

Figure 2.1: Principle of the Total Antioxidant Status assay

Briefly, either 20 μl ddH₂O, standard solution (as provided by the manufacturer) or plasma sample was placed into a disposable cuvette with 1 ml chromagen (metmyoglobin and ABTS®). The samples were mixed and their absorbance read at 600 nm (A₀). Diluted substrate (200 μl, stabilized H₂O₂) was added to the cuvette. The sample was mixed and incubated for exactly 3 min. The final absorbance was measured at 600 nm (A). All procedures were carried out at 37°C. ΔA was calculated for the standard, blank and test samples (ΔA = A - A₀) following which the total antioxidant status was calculated for each test sample using the formula listed below.

\[
\text{Total Antioxidant Status (mM)} = \frac{\text{Antioxidant concentration of standard} \times (\Delta A \text{ blank} - \Delta A \text{ sample})}{(\Delta A \text{ blank} - \Delta A \text{ standard})}
\]
2.2.13 Measurement of hormone levels in plasma

The steroid hormones in plasma were measured with a competitive binding immunoassay using an automated system (Access Immunoassay System Beckman Coulter, Gladesville, NSW). The assay range of the assays for 17β-estradiol, progesterone, cortisol and testosterone were 73-13,216 pmol/L, 0.25-127.2 nmol/L, 11-1655 nmol/L and 0.35-55.5 nmol/L respectively. Cross reactivity with related substances was less than 2% except in the cortisol assay where significant cross reactivity was found with corticosterone (4.7%), Cortisobe (14.5%) and 11-Deoxycorticisol (21.6%). Leptin levels were measured using a competitive ELISA as previously described (Kauter et al., 2000).

2.2.14 Measurement of blood chemistry and haematological values

Blood samples were collected for standard haematological analyses [haemoglobin, white cell counts (WCC), platelets, red cell counts (RCC), haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW), differential white cell count] and for liver function tests (protein, albumin, calc glob, total bilirubin, γ-glutamyltranspeptidase (GGT), alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST)]. These analyses were conducted at the Pathology Laboratory, Armidale and New England Hospital.

2.2.15 Measurement of alkylamide concentrations in plasma

Blood samples were taken 1 h after ingestion of one Echinacea tablet, and were collected into tubes containing lithium heparin. Plasma was separated immediately by centrifugation and stored frozen (-80°C) for subsequent analysis of Echinacea components. Alkylamides were separated from the plasma and concentrated by passage through a solid phase extraction cartridge using methanol as the elution solvent, essentially as previously described (11). Alkylamide concentrations in samples were determined by LC-MS using a gradient HPLC system (Shimadzu LC10AT) coupled to a quadrupole mass spectrometer (Shimadzu) operating in SIM mode (using the molecular ions \( m/z \) according to the masses of the individual compounds) with a positive ion electrospray interface. Caffeic acid derivative concentrations were determined by PDA using a similar HPLC system. The mobile phase for both compound groups was a mixture of water and acetonitrile and was applied using a stepped gradient.
2.2.16 Statistical analysis

Data are presented as the mean ± standard deviation (SD) or alternatively, the mean ± standard error (SE). Statistical analysis of raw data was carried out using the Minitab 13.0 software package (Minitab Inc., USA). Homogeneity of variance was determined using Levene’s test. To determine statistically significant differences, either ANOVA (General Linear Model) or the Student’s t-test was used.