CHAPTER 3: Materials and Methods
Model System

All the experiments were performed on A20 cell line (also known as TIB-208). This is a mouse memory B cell lymphoma line derived from a spontaneous reticulum cell neoplasm found in an old BALB/cAnN mouse. The cells express large amounts of surface IgG2a isotype when grown in RPMI 1640 medium supplemented with 10% FCS.

Materials

RPMI Medium (Invitrogen Life Technologies, Carlsbad, CA)

Per Liter:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI</td>
<td>1 pack</td>
</tr>
<tr>
<td>HEPES</td>
<td>5.96 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.00 g</td>
</tr>
<tr>
<td>Triple distilled milliQ</td>
<td>980 ml</td>
</tr>
</tbody>
</table>

Customized RPMI media was deficient in Lysine and Arginine amino acids and was added separately into the media.

Amino Acids combination used into the media (Sigma Chemical Co., St. Louis, USA)

- L-lysine and L-Arginine
- L-Arginine-¹³C₆ hydrochloride and L-Lysine-¹³C₆ hydrochloride
- L-Lysine-¹³C₆,¹⁵N₂ hydrochloride and L-Arginine-¹³C₆,¹⁵N₄ hydrochloride

To generate double encoding SILAC conditions, normal medium deficient in arginine and lysine was supplemented with Arg[+10] and Lys[+8] for the “heavy” condition, or with Arg[+6] and Lys[+6] for the “medium” condition, or with Arg0 and lys0 for the “light” condition. Final concentrations of arginine and lysine are 84 mg/l and 49mg/l respectively in RPMI. The medium was filtered through 0.25
\( \mu \)m filter. In each SILAC condition, medium was supplemented with 10% dialyzed fetal bovine serum with 10 kDa cutoff membrane and streptomycin/penicillin antibiotic at 10,000 U/ml (Invitrogen Life Sciences, Carlsbad, CA).

**Chemicals, Media Components, Kits and Other consumables**

Chemicals used in the present investigation and their sources are listed in the Table1 below.

**Table1** - List of chemicals and bio-chemicals used in the present study

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Immobilin dry-strips</td>
<td>Amersham Biosciences,</td>
</tr>
<tr>
<td>dithiothreitol (DTT)</td>
<td>Merck (India) Ltd., Mumbai</td>
</tr>
<tr>
<td>Iodoacetamide (IAA)</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Carbinol</td>
<td>Spectrochem Pvt. Ltd, India</td>
</tr>
<tr>
<td>Ammonia water</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>J.T.Baker, Philipsburg, USA</td>
</tr>
<tr>
<td>Anti IgG F(ab)_2</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Disodium EDTA</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>J.T Baker, Philipsburg, USA</td>
</tr>
</tbody>
</table>

**Special materials**

Falcon tubes (15, 50 ml): Greiner, Kremsmünster
Nylon membrane Hybond-(N+): Qiagen, Hilden
Pipettes: Gilson, Hamburg
Protran Nitrocellulose Membrane: Schleicher & Schuell, Dassel
Sterile filter (0.2; 0.45 μm): Millipore, France
X-ray film BioMax MR: Kodak, USA

Reaction sets (Kits)

ECL Western Blot Detection Kit Amersham Biosciences, Freiburg
Phosphoprotein Enrichment Kit, Thermo Scientific, Rockford

Phospho-specific antibodies from Cell Signaling technologies (Beverly, MA, USA):

The target phosphorylation sites were selected from information available in the literature, and were based on the fact phosphorylations at these sites have been implicated in the activation/ altered functioning of the corresponding molecule.

Table 2:

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Phosphorylation Site</th>
<th>Phosphorylation Site</th>
<th>References</th>
</tr>
</thead>
</table>
Composition of Common Media and Solutions

1(a) Buffers and solutions for western blotting

Lysis Buffer for preparation of cytoplasmic extract: 20 mM HEPES, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5% Triton X-100, 0.5 mM DTT, 1 mM sodium orthovanadate, 1 mM NaF, and a cocktail of protease inhibitors

2X SDS-PAGE Buffer: 100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 4% β-mercaptoethanol, 0.01% bromophenol blue

Transfer buffer: 25 mM Tris base, 250 mM Glycine, 0.01% SDS (Sodium Dodecyl Sulfate) and 20% Methanol.

1X PBS (Phosphate-Buffered Saline): 13.7 mM NaCl, 0.27 mM KCl, 10 mM diSodium hydrogen phosphate (Na₂HPO₄) and 0.2 mM Potassium di-hydrogen phosphate (KH₂PO₄).

Blocking buffer: 5% (w/v) Bovine Serum Albumin in 1X PBS-T.

Wash buffer: 0.1% (v/v) Tween-20 in 1X PBS
Primary and secondary antibodies are prepared in wash buffer and blots were developed by enhanced Chemiluminescence (ECL) kit.

1(b) Buffers and solutions for 2D-gel electrophoresis

**Lysis buffer:** 8M Urea, 4% (w/v) CHAPS and 2% (v/v) Pharmalyte (pI 4 – 7).

**Rehydration buffer:** 8M Urea, 2% (w/v) CHAPS, 0.28% (w/v) DTT, 0.1% Bromophenol blue and 0.5% (v/v) Pharmalyte (pI: 4 – 7).

**Equilibration buffer:** 50mM Tris-HCl (pH-8.8), 6M Urea, 30% (v/v) Glycerol, 2% (w/v) SDS and 0.1% Bromophenol blue. Prior to use DTT (10 mg/ml) and Iodoacetamide (25 mg/ml) are added.

**Ponceau-S Stain:** 0.2% w/v Ponceau-S, 3% w/v tri-chloroacetic acid, 3% (w/v) sulfosalicylic acid

**Reagents for Silver Staining:**

Fixation solution (50:5:45 methanol: acetic acid: water), Sensitizing solution (0.02 % sodium thiosulfate), 0.1% AgNO₃, Developing solution (0.04 % formaldehyde in 2 % sodium carbonate), Stopping Solution (1.4 % (w/v) disodium EDTA)
**EXPERIMENTAL PROCEDURES**

**Stimulation of cells and detection of phosphoproteins**

A20 cells (1 x 10^7/ml) were stimulated with the F(ab)_2 fragment of goat anti-mouse IgG at a final concentration of 25 μg/ml in RPMI for a period of up to 30 min. At appropriate times aliquots of cells were collected, centrifuged, and the cell pellets stored in liquid nitrogen. Just prior to electrophoresis, cells were lysed in lysis buffer (20mM HEPES, 10mM NaCl, 1.5mM MgCl₂, 0.2mM EDTA, 0.5% Triton X-100, 0.5mM DTT, 1 mM sodium orthovanadate, 1 mM NaF, and a cocktail of protease inhibitors) followed by removal of the nuclear material and other debris through centrifugation. The detergent soluble proteins were then resolved by SDS-PAGE. Mini Transblot Electrophoretic Cell (Hoefer) was used to transfer the proteins from gel onto nitrocellulose membrane. The apparatus for electroblotting was assembled according to the manufacturer’s instructions. Electroblotting was performed in the presence of 39 mM glycine, 48 mM Tris base, 0.037% SDS and 20% methanol at a constant voltage of 50 V for 1 h at room temperature. The membrane was incubated in odyssey blocking buffer for 2 hr with gentle shaking at 37°C. The blocking buffer was replaced with an appropriate dilution of primary antibody in odyssey buffer with 10% PBS and incubation was continued at 4°C over night with gentle shaking. Specific proteins and phospho-proteins (all phospho-specific antibodies, as described in Table 2, were from Cell Signaling Technologies) were detected. Thereafter, the blots were washed thrice with PBST for 5 min each. After washing, the blots were incubated with 700 nm Goat anti Rabbit odyssey secondary antibody solution (1:10,000...
dilutions in having 10 % PBS) at 37°C for 2 h. The blots were washed as described above, then scanned and analyzed with Odessey Infrared Imaging System.

The values were normalized to loading control (GAPDH and PLC\(\gamma\)2 molecule) on excel.

**siRNA-mediated depletion of adaptor protein**

All the specific siRNAs were procured from Santa Cruz Biotechnology Inc. HiPerfect (Qiagen, Hilden) was used for transfection of cells with the siRNAs (at a final concentration of 100 nM) strictly following the protocol supplied by the manufacturer. In initial standardization experiments, the silencing obtained was between 70-95% at 36 hr after transfection, as detected by Western blotting. For all of the experiments described here, a parallel control set was always included wherein cells were treated with Hiperfect only.

**Radioactive Labeling of cells**

The cells were cultured in phosphate free RPMI for 3-5 hrs after washing properly followed by addition of \(^{32}\)P-labeled orthophosphoric acid (1mCi/ml) for another 2-5 hrs depending upon the radioactive counts in the supplied radioactive compound.

**2-Dimensional Gel Electrophoresis and analysis**

A20 cells (2 x 10\(^7\)) were placed in phosphate-free RPMI supplemented with 1% FCS for 2 hr, following which \(^{32}\)P-orthophosphoric acid (0.5 mCi/ml) was added and the culture continued for an additional 4-5 hrs. They were then stimulated with the F(ab)\(_2\) fragment of goat anti-mouse IgG at a final
concentration of 25 μg/ml for the indicated times. Where necessary, the cells were silenced 36hrs prior to experiment at final concentrations of 100nM of SiRNA. The cytoplasmic fractions of cell lysates were then resolved by 2-DE as previously described (Gorg et al. 2000). Resolution in the first dimension was achieved using 13 cm IPG dry strips (pH, 4-7), and a 12% SDS polyacrylamide gel was used to resolve in the second dimension. Comparable loading in all the groups was ensured through silver staining of the resultant gels. Dried gels were then exposed to X-ray films.

Phosphoproteins were visualized by autoradiography and digitized on a Molecular Dynamics computing densitometer using the ImageQuant software (Amersham Biosciences). Only those spots with an area greater that 75 pixels were considered, and the minimum intensity surrounding the spot on the film was taken as its background and subtracted to give the true intensity. Relative quantification was achieved by normalizing against three distinct spots that were unaffected upon anti-IgG stimulation of cells. Calibration for the Mw and pi was done on the basis of standard markers that were run on parallel gels.

**Silver Staining of polyacrylamide gels**

After the gel has been run, the protein was fixed by incubating the gel slab in fixation solution for 20 - 30 minutes. The gel slab was rinsed with water (2 changes, two minutes per change) and then left it further in water for one hour on a shaking platform. Extended washing was done to eliminate yellowish background usually observed after long developing of the gel. The gel was sensitized with sensitizing solution for 1 - 2 minutes and the solution discarded
with a quick rinse of the gel slab with two changes of water (10 seconds each). The gel was covered with chilled 0.1% AgNO₃ for 30 minutes at room temperature. Silver nitrate solution was discarded and the gel was quickly rinsed with two changes of water (30 seconds per each change). The gel was developed with developing solution and as soon as it turns yellow, it is replaced with a fresh solution. When a sufficient degree of staining has been obtained, it is quenched by discarding the developing solution and replacement with 1.4% (w/v) Na₂EDTA solution for 10 minutes. The gel is washed with MQ water several times and stored in that.

**Cell culture for *in vivo* labeling with SILAC labels**

Three populations of cells are grown in three separate medium formulations containing three isotopically distinct forms of Lysine and Arginine, the light medium containing the amino acid with the natural isotope abundance and the heavy medium containing the two different variants of Lysine and Arginine. Cells are cultured in heavy medium for at least five cell doublings to allow the incorporation of the heavy amino acid. After five cell doublings, 97% of the proteins should be present in the heavy state \((1\left(\frac{1}{2}\right)^{5})\) in incubated cell line. The three different formulations are as follows

<table>
<thead>
<tr>
<th>Normal light medium group1</th>
<th>¹²C₆ Lysine, ¹²C₆Arginine</th>
</tr>
</thead>
<tbody>
<tr>
<td>SILAC labels used in group2</td>
<td>¹³C₆ Lysine, ¹³C₆Arginine</td>
</tr>
<tr>
<td>SILAC labels used in group3</td>
<td>¹³C₆ ¹⁵N₂Lysine, ¹³C₆¹⁵N₄Arginine</td>
</tr>
</tbody>
</table>
In all the media formulation the concentration of Lysine was same as present in normal RPMI media (.280mM). However, the concentration of Arginine was reduced to one fourth (1/4th) the actual concentration (.274mM) in RPMI as Arginine tends to get converted to Proline when in excess. This would interfere with the analysis if heavy Arginine got converted to proline.

**Cell harvesting and lysis**

Serum deprived A20 cells labeled with either L-arginine and L-lysine, L-arginine\textsuperscript{13C6} and L-lysine\textsuperscript{13C6} or L-arginine\textsuperscript{13C6-15N4} and L-lysine\textsuperscript{13C6-15N2} (7 X T75 flasks per condition; ~ 95% confluent cells) were treated with 25\textmu g/ml F(ab)\textsubscript{2} for 0 min, 5 min and 1 min. A second identically labeled set of A20 cells was stimulated with F(ab)\textsubscript{2} for 10 min, 5 min and 20 min, respectively. And the third set was generated for 5 min and 30 minute. All treatments were at 37\textdegree C. Cells were frozen in liquid nitrogen at each time point. After that cells were lysed in lysis buffer (provided by Pierce phosphoprotein enrichment kit) with 10\% CHAPS and 1 mM sodium ortho-vanadate, 5mM NaF, 5mM PMSF, 1mM aprotinin, 1mM leupeptin, 1mM pepstatin and 1mM DTT. The cell-suspensions were mixed 1:1:1 prior to fractionation. The 5 min stimulated cells served as a common reference point in this multiplexing, thus the cell suspensions from this condition were pooled and divided into three equal lots for mixing with the corresponding samples.

Now all the three pooled lysates were placed in ice for 45\textdegree C with vortexing periodically. Thereafter, lysed cells were centrifuges at 10,000g for 20minutes at 4\textdegree C to pellet cellular debris. Supernatant was collected and protein concentration was estimated by Bradford assay to adjust the final concentration to .5mg/ml with lysis buffer.
Phosphoprotein enrichment (Nilsson et al.)

Phosphoprotein Isolation Kit (WGA, PIERCE, cat# 89805) was used to purify phosphoproteins according to the manufacturer's instructions. Shortly, Phosphoprotein Purification Column was equilibrated with 4 ml Phosphoprotein Lysis Buffer without CHAPS. Resin slurry was shaken vigorously and transferred into a spin column and washed with 1 ml of 1×Binding/Washing buffer four times. Then diluted protein sample (.5mg/ml) was added to the resin and incubated for 30 minutes at 4°C on rotator. Afterwards, the column was centrifuged for 1 minute at 1000 × g, and the flow-through was collected. Then the column was washed with 1 ml of 1×Binding/Washing buffer four times. To elute protein, 1 ml of Elution buffer was added into the column and mixed for 10 minutes at 4°C. The column was placed on a 50 ml falcon tube, centrifuged at 1000 × g for 1 minute, and then the eluted fraction was saved. The elution step was repeated five times and the eluted fractions were combined together. The sample volume was reduced to ~100 μl by using centricon of 10kda cutoff (Millipore Corporation, Billerica, MA).

Tryptic digestion

The total enriched phosphoprotein was estimated using microBradford Assay. Thereafter, sample was lyophilized and resuspended in dissolution buffer supplied by Applied Biosystems trypsin digestion kit. The digestion was carried out according to manufacturer's instructions. Briefly explained, the lyophilized sample was dissolved in 80 μl of Dissolution buffer supplied from the Applied Biosystems trypsin digestion kit (www.appliedbiosystems.com), denatured and
vortexed. Thereafter, 2 μl of reducing agent (Applied Biosystems/MDS Sciex, Germany) was added followed by incubation for 30 min at 60°C. The sample was kept at room temperature to be cooled. After short spin, cysteine-blocking agent was added and incubated for 10 min at room temperature. Now trypsin (modified sequencing grade; Promega, Madison, WI) was mixed at 1:50 ratio (enzyme: substrate) and incubated for 16 h at 37°C at pH 8.0.

**Strong Cation Exchange Chromatography (Gruhler et al., 2005)**

The SILAC labeled peptide pool was resuspended in 170 μl of buffer A (5 mM ammonium formate, 30% (v/v) acetonitrile, pH 2.9) and separated on a strong cation exchange Zorbax 300 SCX column (PolySULFOETHYL Aspartamide) with dimensions 150 x 2.1 mm, 3 μm, 300 Å (Agilent technologies, Columbia, MD) under the following conditions: flow rate 400 μl/min, BufferB (500 mM ammonium formate, 30% acetonitrile, pH 2.9) 20-min gradient, 0–30% B (A + 500 mM ammonium formate, 30% acetonitrile, pH 2.9); 10 min, 60–100% B; 10 min, 100% B. One minute fractions were collected in eppendorfs manually and vacuum-dried.

**LC-ESI-Q-TOF MS/MS**

LC-MS/MS was performed with a nanoflow LC (1100 series, Agilent Technologies, USA and ZORBAX 300SB C18 column) coupled to a QSTAR XL, ESI-hybrid Q-TOF tandem mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada). A Zorbax BioSCX series 3.5μm, 0.3 x 35 mm (Agilent Technologies, DE, Germany) was used as trap column for preliminary sample
clean up and 3.5μm, 75μm x 150 mm ZorbaxSB C18 material based analytical column (Agilent Technologies, DE, Germany) was used for peptide separation. The mobile phases were 3% ACN/0.1% HCOOH (A) and 90% ACN/0.1% HCOOH (B). LC gradient elution condition was initially 1% B to 5% B (5 min), 5-25% B (35 min), 25-50% B (25 min), 50-85% B (15 min), 85-90% B (5 min) with a flow rate of 250 nL/min. The program AnalystQS (version 1.1; Applied Biosystems) was used for data acquisition and instrument control. Information dependent data acquisition (IDA) was performed in positive ion mode. Three of the most intense precursor ions doubly or triply charged was selected from \( m/z \) 350 to \( m/z \) 1600 from MS scan. MS/MS spectra generated with CID were acquired for 4s from \( m/z \) 100 to \( m/z \) 2000. The collision energy (CE) was automatically calculated based upon peptide charge and mass to charge ratio \( (m/z) \); CE against doubly charged peptide was 0.0625 x \( m/z \) - 3 (V), and CE against triply charged peptide was 0.0625 x \( m/z \) - 5 (V). Maximum allowed CE was set to 80 V. A dynamic exclusion window was applied which prevented the same \( m/z \) from being selected for 90 sec after its acquisition.

**Analysis of the Spectra and Extraction of Phosphoproteins**

The MS/MS spectra were extracted and searched against Uniprot-sprot database (version 51.5, total number of entries 230093, entries for *Mus musculus* 14515) using ProteinPilot™ software (version 3.0, revision 33087, Applied Biosystems) with the Paragon™ method utilizing the following search parameters: *Mus musculus* as species, trypsin as enzyme as specificity, cysteine static modification with methylmethanethiosulfate and SILAC (peptide labeled at N
terminus and lysine) as sample type with phosphorylation emphasis and gel based id. The raw peptide identification results from the Paragon™ Algorithm (Applied Biosystems) searches were further processed by the Pro Group™ Algorithm (Applied Biosystems) within the Protein Pilot software before final display (Shilov et al., 2007). Non-redundant phosphopeptides and proteins with descriptors of "unnamed or unknown" were removed.

The Pro Group Algorithm uses the peptide identification results to determine the minimal set of proteins that can be reported for a given protein confidence threshold. For each protein, Pro Group Algorithm reports two types of scores: unused ProtScore and total ProtScore. The total ProtScore is a measurement of all the peptide evidence for a protein and is analogous to protein scores reported by other protein identification softwares. The unused ProtScore, however, is a measurement of all the peptides evidence for a protein that is not better explained by a higher ranking protein. In other words, the unused ProtScore is calculated by using the unique peptides (peptides that are not used by the higher ranking protein), and it is a true indicator of protein evidence. This is how a single-protein member of a multiprotein family has been singled out.

False Discovery Rate (FDR) analysis was also performed. This analysis is performed by the ProteomicS PEP Software (referred to as “PSPEP”) that is installed with ProteinPilot Software. Because the false discovery rate analysis is independent of the statistics reported by ProteinPilot Software, PSPEP provides an independent way to determine the quality of your identification results.

The general approach used by PSPEP is called “decoy database searching”. The principle of decoy searching is to search a collection of answers that are
known to be wrong – "decoy" proteins – in addition to the database of interest – "target" proteins (Tang et al., 2008). The relative rate of reporting of identifications from the database of interest versus the known incorrect answers indicates the likelihood that wrong answers are reported from the database of interest. To generate a sufficient number of wrong answers, the software was set to the Detected Protein Threshold to 0.05.

The protein confidence threshold cutoff for this study was ProtScore 2.0 (unused) with at least one peptide with 99% confidence. The mean, standard deviation, and $p$ values to estimate statistical significance of the protein changes were calculated by Pro Group.

Again, all the phosphoproteins identified by above cut off were further filtered depending upon the literature survey. We listed all the phosphoproteins which passed one of the following criteria,

1. The confidence of the at least one of the phosphopeptide of the same protein must be above 80.
2. Or if the phosphoprotein doesn't have the phosphopeptide with good confidence score, it must be there in the reported literature.

All the phosphoproteins were exported to excel sheet and summarized to extract the information.

**Quantitation of relative phosphoprotein abundance**

All the phosphopeptides searched by the software and passing the check, of each phosphoprotein were taken to quantitate relative ratios at each time point. Minimum peak area threshold of 500 for SILAC quantitation was required,
Relative quantitation of peptide abundance was performed via calculation of selected ion chromatogram (SIC) peak areas of heavy and light SILAC-labeled peptides by the software embedded algorithm with bias correction. Weighted Average of Log Ratios

$$\text{Weighted Average of Log Ratios} = \frac{\sum_{i=1}^{n} w_i \times x_i}{\sum_{i=1}^{n} w_i}$$

Where:

- $x_i = \log \text{(peptide ratio}_i\text{)}$ where “(peptide ratio}_i\text{)” is the ratio for the ith observation;
- $w_i = 1/% \text{ Error}_i$, the weight for the ith observation
- $n$ is the number of peptide ratios contributing to a protein’s average ratio

Heat maps were generated for demonstrating the dynamics of phosphorylation of each observed protein in time dependent manner. Protein was considered to be upregulated if the signal log ratio between the reference and the target samples was higher than one (>2-fold increase) and the detection P-value of the target sample was <0.05. Similarly, a protein was defined as downregulated if the signal log ratio was less than minus one (>2-fold decrease) and the detection P-value of the reference sample was <0.05.
**Gene Ontology analysis**

Cytoscape along with its Plug-in Bingo 2.0182 was used to analyze the distribution of experimental datasets among various protein groups, and to identify significantly overrepresented biological functions of the proteins. The Gene Ontology (GO) annotations of proteins were compared with the ones of a reference proteome (e.g. identified proteins vs. the entire protein database, or a subset of the identified proteins vs. the overall identified proteins). To assign corresponding GO identifiers to each Swiss-Prot entry, the Gene Ontology Annotation database was used. The hypergeometric test and the Benjamini & Hochberg False Discovery Rate correction were performed to derive overrepresented functions (Maere et al., 2005; Shannon et al., 2003). A probability value of 0.05 was considered significant.