Ocimum sanctum leaf extracts stimulate insulin secretion from perfused pancreas, isolated islets and clonal pancreatic β -cells

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Abstract

Ocimum sanctum leaves have previously been reported to reduce blood glucose when administered to rats and humans with diabetes. In the present study, the effects of ethanol extract and five partition fractions of *O. sanctum* leaves were studied on insulin secretion together with an evaluation of their mechanisms of action. The ethanol extract and each of the aqueous, butanol and ethylacetate fractions stimulated insulin secretion from perfused rat pancreas, isolated rat islets and a clonal rat β -cell line in a concentration-dependent manner. The stimulatory effects of ethanol extract and each of these partition fractions were potentiated by glucose, isobutylmethylxanthine, tolbutamide and a depolarizing concentration of KCl.

Inhibition of the secretory effect was observed with diazoxide, verapamil and Ca^{2+} removal. In contrast, the stimulatory effects of the chloroform and hexane partition fractions were associated with decreased cell viability and were unaltered by diazoxide and verapamil. The ethanol extract and the five fractions increased intracellular Ca^{2+} in clonal BRIN-BD11 cells, being partly attenuated by the addition of verapamil. These findings indicated that constituents of *O. sanctum* leaf extracts have stimulatory effects on physiological pathways of insulin secretion which may underlie its reported antidiabetic action.

Journal of Endocrinology (2006) **189**, 127–136

Introduction

Diabetes is a serious metabolic disorder with micro- and macrovascular complications that result in significant morbidity and mortality. The aging population, consumption of calorie-rich diets, obesity and sedentary lifestyles have led to a tremendous increase in the number of individuals with type 2 diabetes worldwide. Current drugs used for diabetes therapy are not free from side effects and do not restore normal glucose homeostasis (Rang *et al.* 1991). Moreover, providing modern medical healthcare across the world, especially in developing countries, is still a far-reaching goal due to economic constraints. Thus, it is necessary to look for new and, if possible, more efficacious drugs and to make use of the vast reserves of phytotherapy for medicinal purposes.

Since time immemorial, individuals with diabetes have been treated orally in folk medicine with a variety of plant extracts. Recently, there has been increasing interest in the use of medicinal plants. The plant kingdom has become a target for multinational drug companies and research institutes for the discovery of new biologically active compounds and potential drugs (Evans 1996). The World Health Organization has recommended, especially in

developing countries, the initiation of programmes designed to use medicinal plants more effectively in the traditional healthcare system (World Health Organization 1978). The resolution of the 31st World Health Organization Assembly requested a complete inventory, and a thorough evaluation of the efficacy, safety and standardization of medicinal plants for the treatment of diabetes (Farnsworth 1980).

Ethnobotanical information indicates that more than 800 plants have been used as traditional remedies for the treatment of diabetes (Ajgaonkar 1979, Alarcon-Aguilara *et al.* 1998). The antihyperglycaemic activity of a large number of these plants has been evaluated and confirmed in different animal models (Karawya *et al.* 1984, Farjou *et al.* 1987, Swanson-Flatt *et al.* 1991a, 1991b, Jouard *et al.* 2000). In India, a number of plants are mentioned in ancient literature (Ayurveda) for the treatment of diabetes and some of them have been tested experimentally (Chopra *et al.* 1956, Rajashekharan & Tuli 1976, Chattopadhyay 1993, 1999, Pugazhenthii & Murthy 1996, Joy & Kuttan 1999).

Ocimum sanctum Linn. (Labiatae), commonly known as holy basil, is an herbaceous plant found throughout the south Asian region. The plant grows wild in India, but is

Figure 1 Preparation of ethanol extraction and five partition fractions (hexane, chloroform, butanol, ethylacetate and aqueous) of dried leaves of *O. sanctum*.

also widely cultivated in homes and temple gardens. Apart from religious significance, it has a long history of medicinal use and is mentioned in *Charak Samhita*, the ancient textbook of Ayurveda. Other texts mention the use of basil leaves for a variety of conditions such as catarrhal bronchitis, bronchial asthma, dysentery, dyspepsia, skin diseases, chronic fever, haemorrhage and helminthiasis, and topically for ring worms (Singh *et al.* 1980, Kirtikar & Basu 1993, Wagner & Winterhoff 1994, Warier 1995a). Fresh leaves taken with black pepper are used as a prophylactic measure for malaria (Dastur 1962). Holy basil has been shown also to be effective as antistress, adaptogenic and attenuates the stress-induced changes (Bhargava & Singh 1981, Singh *et al.* 1991a, 1991b).

Leaves of *O. sanctum* have been shown to possess hypoglycaemic effects in experimental animals (Joglekar *et al.* 1959, Dhar *et al.* 1968, Chattopadhyay 1993, Rai 1997). The ethanol (70%) extract of *O. sanctum* leaves caused a significant reduction of blood glucose in normal, glucose-fed hyperglycaemic and streptozotocin-treated diabetic rats. A diet containing leaf powder (1%) fed to normal and diabetic rats for 1 month significantly reduced fasting blood glucose (Rai 1997). In a randomized, placebo-controlled, crossover, single blind clinical trial, leaf extract of *O. sanctum* caused a significant decrease in fasting and post-prandial glucose (Agrawal *et al.* 1996). More recently, an ethanol extract of *O. sanctum* was shown to reduce hyperglycaemia in alloxan diabetic rats in both acute and long-term feeding studies (Vats *et al.* 2002).

Despite this research, no studies have been performed to assess how the antihyperglycaemic action of *O. sanctum* is mediated. In the present study, an ethanol extract and

five partition fractions of *O. sanctum* leaves were evaluated for their effects on insulin secretion using isolated perfused rat pancreas, isolated islets and clonal BRIN-BD11 cells.

Materials and Methods

Plant material and preparation of extracts and fractions

Dried *O. sanctum* (holy basil) leaves were purchased from Ramkrishna Mission, Kolkata, India, and botanically authenticated. Voucher specimens were deposited in the National Herbarium, Bangladesh. The leaves were dried at 40 °C and ground into a fine powder. The powder (2 kg) was extracted with 80% ethanol (10 l) in a stainless steel extraction tank for approximately 4 days at room temperature by changing ethanol daily. The combined extract was filtered and evaporated to dryness using a rotary evaporator. A membrane pump was used to evacuate the extract in order to remove the residual solvent. The extract was finally freeze dried (275 g) by using a Varian 801 LY-3-TT freeze-dryer (Varian, Lexington, MA, USA). The dry sample was stored at 4 °C.

The ethanol extract (100 g) was subsequently partitioned between hexane (3 × 0.5 l) and water (0.5 l). The hexane fraction was separated and evaporated to dryness to get hexane-soluble material (19.6 g). The aqueous layer was further partitioned (3 × 0.5 l) using solvents and evaporated to dryness to get chloroform- (16.9 g), ethyl acetate- (5.5 g) and butan-1-ol- (7.8 g) soluble materials. The residual aqueous part was condensed by rotary evaporator and finally freeze dried. Figure 1 outlines the

preparation of ethanol extraction and five partition fractions of dried leaves of *O. sanctum*.

Effects of O. sanctum on insulin secretion from perfused pancreas

Long-Evans rats (180–250 g) were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the pancreas was isolated and perfused at 37 °C according to the method of Giroix *et al.* (1983). Extract and fractions of *O. sanctum* were dissolved in Krebs–Ringer bicarbonate buffer (KRB; 118 mM NaCl, 4 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 1.25 g/l BSA and 40 g/l dextran T70, pH 7.4) containing 2.8 or 11.1 mM D-glucose. The perfusate was continuously gassed with a mixture of O₂/CO₂ (95:5). After the first 20-min equilibration period, the composition of the perfusate changed as indicated in Fig. 2 (see below). Effluent samples were stored at –20 °C for insulin assay.

Effects of O. sanctum on insulin secretion from isolated islets

Islets were isolated on the day of experimentation from the pancreas of Long-Evans rats (180–250 g) by collagenase digestion (Moskalewski 1969). Following preincubation for 40 min in KRB containing 3 mM glucose, batches of 8–10 islets were incubated for 1 h at 37 °C in 400 µl buffer containing 3 or 11.1 mM glucose and with either extracts or fractions of *O. sanctum* (detailed in Table 1, see below). Aliquots of supernatant were stored at –20 °C for insulin assay.

Effects of O. sanctum on insulin secretion and intracellular Ca²⁺ in clonal β-cells

Clonal BRIN-BD11 cells were used to evaluate the mechanism underlying the effects of *O. sanctum* on insulin secretion, including modulation of intracellular Ca²⁺. The origin and characteristics of this glucose-responsive rat cell line are described in detail elsewhere (McClenaghan *et al.* 1996). Cells were seeded into 24-well plates 0.15 × 10⁶ cells/well and allowed to attach overnight. Following preincubation for 40 min in KRB supplemented with 1.1 mM glucose, cells were incubated for 20 min at 37 °C in the same buffer supplemented with glucose, *O. sanctum* extracts and other reagents as indicated in the figures. In selected experiments, the viability of cells exposed to various plant extracts was assessed. Aliquots were removed from each well and stored at –20 °C for insulin assay. In further series of experiments, the effects of *O. sanctum* on intracellular Ca²⁺ ([Ca²⁺]_i) were determined using BRIN-BD11 cell monolayers seeded onto 96-well black-walled, clear-bottomed microplates (Greiner). Cells were washed with KRB and loaded with FLEX calcium assay reagent (Molecular Devices,

Sunnyvale, CA, USA), for 10 min at 37 °C (Miguel *et al.* 2004). Fluorometric data during subsequent exposure to plant extract and other test agents were acquired using the FLEXstation™ (Molecular Devices) at a wavelength of 525 nm.

Analysis

For perfusion and islet studies, insulin was measured by ELISA using kits supplied by Crystal Chem (Downers Grove, IL, USA). In studies using BRIN-BD11 cells, insulin was measured by RIA (Flatt & Bailey 1981). The protein content of islets was determined using the detergent-compatible protein kit supplied by BioRad. Cell viability was evaluated by modified Neutral Red assay (Hunt *et al.* 1987).

Statistical analysis

Results are presented as means ± S.D. for a given number of observations (*n*). Data from each set of observations were compared using unpaired Student's unpaired *t*-test and Mann–Whitney *U*-test where appropriate (SPSS for Windows). One-way analysis of variance (ANOVA) was performed and comparisons with the control group made using Dunnett's test to preserve an overall error rate of 5%. Differences were considered significant if *P* < 0.05.

Results

Effects of O. sanctum on insulin secretion from perfused pancreas

Ethanol extract of *O. sanctum* caused a significant (*P* < 0.001) biphasic increase in insulin release during 10-min perfusion, with a peak 21-fold increase above the basal level (Fig. 2A). Subsequent exposure for 5 min to 11 mM glucose caused a steep elevation of insulin release from the basal level of 0.05 ± 0.01 ng/ml to peak value of 1.12 ± 0.08 ng/ml (*P* < 0.001). When extract was reintroduced at 11 mM glucose, there was a further protracted stimulation of insulin release (*P* < 0.05), which reversed the falling insulin output observed in the continued presence of 11 mM glucose alone. As shown in Fig. 2B, perfusion with aqueous, butanol and ethylacetate fractions significantly (*P* < 0.001) increased in insulin release in an almost similar pattern (highest peak of insulin secretion: basal, 0.03 ± 0.01 ng/ml; aqueous fraction, 1.7 ± 0.2 ng/ml; butanol fraction; 1.2 ± 0.1 ng/ml; ethylacetate fraction; 1.4 ± 0.1 ng/ml). These fractions stimulated further insulin release at 11.1 mM glucose (peak output: 1.33 ± 0.1 versus 1.8 ± 0.1, 1.3 ± 0.1, 1.6 ± 0.1, 1.2 ± 0.1 and 1.5 ± 0.1 ng/ml, respectively; *P* < 0.05). Hexane and chloroform fractions showed less prominent effects on insulin

Figure 2 Effects of the (A) ethanol extract and (B) five partitioned fractions of *O. sanctum* on insulin release from perfused rat pancreas. Data are means $\pm$ s.d. from four or five separate experiments. Pancreas was perfused with extract/fractions at a dose of 1 mg/min. The glucose concentration was raised from the basal level of 2.8 mM to 11 mM. EtOH, ethanol; Ext, extract; Fr, fraction; Gn, glucose plus concentration in mM; Hex, hexane; Chlor, chloroform; EtAc, ethylacetate; But, butanol; Aq, aqueous.

Table 1 Effects of the ethanol extract and five partition fractions of *O. sanctum* on insulin secretion from isolated rat islets

Group	Yield from starting materials (g/kg)	Insulin secretion (ng/mg islet protein)	
		3 mM Glucose	11 mM Glucose
Control (n=6)	No extract/fraction	2.99 (2.65–4.27)	5.41 (4.91–9.27)
Ethanol (80%) extract (n=6)	137.5	4.82 (3.85–6.20)*	6.23 (4.95–8.49)*
Ethylacetate fraction (n=7)	7.6	5.41 (3.2–8.13)**	6.15 (3.41–11.25)*
Butanol fraction (n=6)	10.8	5.96 (4.90–10.32)**	7.68 (5.46–12.29)*
Aqueous fraction (n=6)	66.2	4.98 (3.36–9.24)*	6.49 (3.46–8.69)*
Hexane fraction (n=6)	27.0	3.08 (2.67–4.91)	5.49 (4.78–7.58)
Chloroform fraction (n=6)	23.2	3.19 (2.42–5.98)	5.46 (4.51–9.12)

Isolated rat islets were incubated for 60 min with ethanol extract and partition fractions of *O. sanctum* (30 µg/ml) in the absence and presence of 11 mM glucose. Data are presented as medians, with ranges in parentheses. Mann–Whitney *U*-test was used to evaluate statistical significance. **P*<0.05, *P*<0.01 compared to control.

release from perfused pancreas, especially at the lower glucose concentration (Fig. 2B).

Effects of *O. sanctum* on insulin secretion from isolated islets

Table 1 shows the effects of the ethanol extract and five partition fractions on insulin secretion from isolated rat islets in the presence of 3 and 11.1 mM glucose. Ethanol extract and three partition fractions (ethyl acetate, butanol and aqueous) increased insulin release by 61–99% compared with the 3 mM glucose control (*P*<0.05 and *P*<0.01; Table 1). Increasing glucose concentration from 3 to 11.1 mM caused a 2-fold increase in insulin release. The ethanol extract and three fractions (ethyl acetate, butanol and aqueous) enhanced glucose-induced insulin secretion by 1.2–1.4-fold when compared with the 11.1 mM glucose control (*P*<0.05). Hexane and chloroform fractions lacked significant effects on insulin release.

Effects of *O. sanctum* on insulin secretion from clonal BRIN-BD11 cell line

The effects of increasing concentrations (8–5000 µg/ml) of ethanol extract and five partition fractions of *O. sanctum* on insulin secretion from BRIN-BD11 cells are shown in Fig. 3. The ethanol extract evoked concentration-dependent 2–22-fold increases in insulin release compared with 5.6 mM glucose alone (*P*<0.05–*P*<0.001). Concentrations up to 200 µg/ml did not alter cellular viability but higher concentrations decreased viability by 15–20% (data not shown). Three fractions of *O. sanctum* (aqueous, butanol and ethyl acetate) stimulated insulin secretion in a concentration-dependent manner between 200 and 5000 µg/ml (*P*<0.05–*P*<0.001). Only at the higher concentration of 5000 µg/ml was cell viability decreased (aqueous, 87 ± 15%, *P*<0.05; butanol, 85 ± 12%, *P*<0.05; ethylacetate, 78 ± 13%, *P*<0.01). Chloroform

and hexane fractions increased insulin release (*P*<0.001) at 5000 µg/ml, but this was associated with a significant reduction of cell viability (chloroform, 37 ± 6.0%; hexane, 50 ± 10%; *P*<0.001).

Further studies used non-toxic concentrations to evaluate the possible mechanisms underlying the insulin-secretory actions of *O. sanctum*. As shown in Fig. 4, insulin release induced by 200 µg/ml ethanol extract (*P*<0.001) was potentiated by 16.7 mM glucose (*P*<0.05), isobutylmethylxanthine (IBMX; *P*<0.001) and tolbutamide (*P*<0.05). In contrast, the effects of *O. sanctum* extracts were inhibited by diazoxide (*P*<0.01) and verapamil (*P*<0.001). The ethanol extract also maintained its ability to enhance insulin secretion from cells depolarized with 30 mM KCl (Fig. 4). Similar effects were noted for aqueous, butanol and ethylacetate fractions in the presence of insulin secretagogues (glucose, IBMX, tolbutamide), inhibitors (diazoxide and verapamil) and depolarizing concentrations of KCl (Fig. 4). Removal of Ca²⁺ from the test buffer significantly reduced (*P*<0.001) but did not totally abolish insulin release induced by ethanol extract and three partition fractions (aqueous, butanol and ethylacetate; *P*<0.001, Fig. 5).

Effects of *O. sanctum* on intracellular calcium in the clonal BRIN-BD11 cell line

Figure 6 shows the effects of the ethanol extract and three partition fractions (aqueous, butanol and ethylacetate) of *O. sanctum* on [Ca²⁺]_i. Exposure to the ethanol extract and both the aqueous and butanol fractions produced a prompt increase of ([Ca²⁺]_i). This was largely reduced by the addition of the blocker of voltage-dependent calcium channels, verapamil (50 µM). Similar effects were observed with the ethyl acetate fraction but effects were much less prominent (Fig. 6).

Figure 3 Effects of different concentrations of the ethanol extract and five partition fractions of *O. sanctum* on insulin release from BRIN-BD11 cells. Results are means $\pm$ s.d. from eight separate observations. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with 5.6 mM glucose. One-way ANOVA was performed and pairwise comparisons with the control (5.6 mM glucose) group used Dunnett's test to preserve an overall error rate of 5%.

Discussion

The leaves of *O. sanctum* have been claimed to possess antidiabetic properties by the traditional healers. There exist

some scientific reports (Agarwal *et al.* 1996, Rai 1997, Chattopadhyay 1999) consistent with the anti-hyperglycaemic activity of this plant, but its mechanism of action has not yet been elucidated. This study has

Figure 4 Effects of the ethanol extract and five partition fractions of *O. sanctum* on insulin release from BRIN-BD11 cells in the presence of established stimulants and inhibitors of insulin secretion. Results are means $\pm$ s.d. from eight separate observations. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with 5.6 mM glucose in the presence or absence of plant extract. $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ compared with the respective incubations in the absence of plant extract. +++ $P < 0.001$ compared with 16.7 mM glucose in the presence or absence of plant extract. One-way ANOVA was performed and pairwise comparisons with the control (5.6 mM glucose) used Dunnett's test to preserve an overall error rate of 5%. dia, diazoxide; G, glucose; Tol, tolbutamide; vera, Verapamil.

examined the insulinotropic effects of *O. sanctum* using the perfused rat pancreas, isolated rat islets and the clonal rat BRIN-BD11 cell line.

Prominent insulin-secretory effects of the ethanol extract and three partition (ethylacetate, butanol and aqueous) fractions were noted in the perfused rat pancreas. Similar effects were found in acute insulin-release studies using isolated rat islets and the clonal rat cell line BRIN-BD11. In these experiments, prominent insulinotropic effects were observed in the more polar compared with the hexane and chloroform fractions. Ethanol extract and the aqueous, butanol and ethylacetate fractions showed dose-dependent stimulatory effects on insulin secretion in BRIN-BD11 cells. Hexane and

chloroform fractions were effective at higher concentrations, but this was associated with toxic effects on cell viability.

Non-toxic concentrations of *O. sanctum* extract and partition fractions were used to study mechanisms underlying stimulation of insulin secretion. The extract stimulated basal insulin secretion with an action enhanced by increasing the glucose concentration over the range 2.8–16.7 mM. The effects of tolbutamide, and a membrane-depolarizing concentration of KCl (30 mM), were tested in the absence and presence of the extract. This sulphonylurea is known to act by closing K_{ATP} channels, depolarizing the plasma membrane and stimulating Ca^{2+} entry by activation of voltage-dependent

Figure 5 Effects of various concentrations of the ethanol extract and three partition fractions of *O. sanctum* on insulin release from BRIN-BD11 cells in the presence and absence of Ca^{2+} . Results are means $\pm$ S.D. from eight separate observations. *** $P < 0.001$ compared with 5.6 mM glucose in the presence of Ca^{2+} . $\Delta\Delta\Delta P < 0.001$ compared with the respective concentration in the presence of Ca^{2+} . +++ $P < 0.001$ compared with 5.6 mM glucose in the absence of Ca^{2+} . One-way ANOVA was performed and pairwise comparisons with the control (5.6 mM glucose) group used Dunnett's test to preserve an overall error rate of 5%. Eth ext, ethanol extract; Aq fr, aqueous fraction; Bu fr, butanol fraction; EthAc fr, ethylacetate fraction.

Figure 6 Effects of the ethanol extract and three partition fractions of *O. sanctum* on $[\text{Ca}^{2+}]_i$ in BRIN-BD11 cells in the absence and presence of verapamil. Each data point represents the mean $\pm$ S.D. from six separate observations. RFU, relative fluorescence units.

calcium channels (Boyd 1988). The extract enhanced insulin secretion under both conditions, indicating that the extract acts by mechanisms in addition to effects on K_{ATP} and other ion channels. Augmentation of insulin secretion in these depolarized conditions suggests possible intracellular actions such as stimulation of adenylate cyclase/cAMP or the phosphatidylinositol pathway, or direct effects on exocytosis.

Additional experiments were performed to assess the effects of extract on insulin secretion in the presence of other modulators, known to affect secondary-messenger pathways in β -cells. The effects of IBMX, an inhibitor of cAMP phosphodiesterase (Sharp 1979), were markedly increased by *O. sanctum*. Interestingly, the traditional use of *O. sanctum* for treatment of asthma in India has been scientifically supported (Singh *et al.* 1980, Sharma 1983, Palit *et al.* 1983, Warier 1995b). The anti-asthmatic actions have been attributed to elevation of cAMP in bronchial smooth muscle cells, promoting airway relaxation and blocking replication of smooth muscle cells (Tomlinson *et al.* 1995).

Involvement of ion channels in the stimulatory action of *O. sanctum* was also evident in β -cells. Diazoxide, a K_{ATP} -channel opener (Henquin *et al.* 1992), inhibited the

insulin-releasing effects of the extract, suggesting that closure of K_{ATP} channels participates in the overall mechanism of action of *O. sanctum*. This is supported by observations with verapamil, a voltage-dependent Ca^{2+} channel blocker (Weinhaus *et al.* 1995) that abolished the insulin-releasing effects of the extract. Similar dependence on extracellular Ca^{2+} was evident from experiments testing the effects of Ca^{2+} -free buffer. The omission of Ca^{2+} did not completely abolish the insulin-secretory effects, suggesting that the extract can induce mobilization of intracellular Ca^{2+} as well as promoting Ca^{2+} entry. This conclusion was well supported by direct observation of intracellular Ca^{2+} in BRIN-BD11 cells. The extract had a rapid and prominent stimulatory effect on $[Ca^{2+}]_i$ which was inhibited but not abolished by verapamil.

Interestingly, each of the partition (aqueous, butanol, ethylacetate) fractions of *O. sanctum* exerted broadly similar effects on insulin secretion to the ethanol extract, including responses to insulinotropic antagonists (diazoxide, verapamil), agonists (tolbutamide, IBMX), depolarizing conditions (30 mM KCl plus 16.7 mM glucose) and Ca^{2+} depletion, and also on $[Ca^{2+}]_i$. This reveals that the ethanol extract and these fractions contain active molecules that exert effects on the β -cells via similar mechanisms. Constituents of *O. sanctum* extracted by these polar solvents include polysaccharides, glycosides, amino acids, flavenoids and terpinoids (Platel & Srinivasan 2000). Such activity was lost in the hexane and chloroform partition fractions.

In conclusion, this study has shown that *O. sanctum* leaf extracts exert prominent stimulatory effects on insulin secretion from the β -cells via physiological pathways. *In vivo* studies also indicate that the ethanol extract decreased blood glucose and increased plasma insulin in type 2 diabetic rats (Hannan *et al.* 2003). Future work directed at the purification and characterization of active components may reveal new agents for diabetes therapy.

Acknowledgement

These studies were supported in part by University of Ulster Research Strategy Funding. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 19 December 2005

Accepted 6 January 2006

Made available online as an Accepted Preprint
19 January 2006