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Foreword from the Editor

Dear JUMMEC readers,

Now that we've reached the midpoint of 2016, it is a good time to look back and reflect on the progress and achievements in the fields of medicine and healthcare. It is also a good opportunity to look forward to the future and identify key areas of medical research that would benefit not only the citizens of our country but also the world. As it is, there are many key issues that are currently highlighted as areas of concern including public opinions on vaccination and the rise of drug-resistance against many different pathogens. For this issue of JUMMEC (Issue 1, Volume 19) it is my pleasure to introduce the contributions of a few authors in the fields of Health and Translational Research.

Major posterior approach spinal surgery is a complicated procedure that requires care and also prevention of hypothermia. Dr. Ina Ismiarti Shariffuddin and her team present a study comparing the efficiency of under-body forced-air warming versus a resistive heating blanket for prevention of hypothermia during spinal surgery indicating that the former technique is superior to the latter in terms of prevention. Hasani *et al* on the other hand present an interesting study on aloe emodin extracted from the leaves of Aloe barbadensis Miller which enhances the cytotoxic effects of tamoxifen on to ERα-positive breast cancer cells (MCF-7) through apoptosis.

Living donation is an important source for kidney transplantations in Malaysia and Makmor *et al* have looked into primary data that aims to investigate the follow-up attendance, financial circumstances of the follow-up cost, and preference of medical-institutional facilities of Malaysian kidney donors. The team provides good data that helps healthcare providers identify the chief concerns of Malaysian living organ donors. Another study in this issue looks done by Ramasamy *et al* review stem cell therapy as a potential treatment for glaucoma. In this review, nine studies were selected and the results of these studies showed that there was a potential in stem cell based therapy in treating glaucoma.

Finally, we have a case report by Dr. Yong-Muh Ng involving a patient with relapsed culture-proven *Salmonella typhi* infection who developed septic shock and subsequently Parkinsonism. A lumbar puncture had revealed acellular cerebrospinal fluid with raised protein level and magnetic resonance imaging revealed cerebral petechial haemorrhages resulted from small vessels vasculitis.

We would like to thank the readers and contributors to JUMMEC as well as the continued support from Faculty of Medicine, University Malaya as this has allowed JUMMEC to continually provide quality review articles and published material of relevance to scientists and healthcare professional worldwide. This sharing of knowledge will go a long way to improving research in the fields of health and medicine.

Lau Yee Ling
Managing Editor (Volume 19 Issue 1)

UNDER-BODY FORCED-AIR WARMING BLANKET VERSUS RESISTIVE HEATING BLANKET FOR PREVENTION OF HYPOTHERMIA DURING SPINAL SURGERY: A RANDOMIZED PROSPECTIVE STUDY

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ABSTRACT

Background:

Prevention of hypothermia in patients undergoing major posterior approach spinal surgery can be difficult, as a large body surface is exposed to the cold environment of the operating theatre. We compared the efficacy of a new under-body forced-air warming blanket with that of a resistive heating blanket in preventing hypothermia.

Methods:

Sixty patients undergoing major posterior approach spinal surgery lasting for more than 2 hours were randomly assigned to warming with a full under-body forced-air warming blanket or three segments of resistive heating blankets, both set at 42°C. The ambient temperature was kept near 20°C. Nasopharyngeal, rectal and axillary temperatures were measured at regular intervals. Changes in core temperature (average of nasopharyngeal and rectal) over time were compared by the independent t-test.

Results:

The characteristics of the patients were comparable. The baseline core temperature was 36.36 ±0.38°C in the forced-air group and 36.27 ± 0.46°C in the resistive heating group. During the first hour, the core temperature decreased similarly from baseline in both groups. From 100 minutes after induction until the end of the surgery, core temperature rose in both groups. At the end of surgery, the core temperature was increased by 0.08 ± 0.09°C from baseline in the forced-air group but decreased by 0.40 ±0.04°C from baseline in the resistive heating group. The difference in the change of the core temperature, at the end of the surgery, between the two groups is statistically significant ($P<0.05$).

Conclusion:

We demonstrated that the new under-body forced-air warming blanket is superior to the resistive heating blanket in preventing hypothermia in patients undergoing major posterior approach spinal surgery.

***Keywords**: forced-air warming, resistive heating, blanket, hypothermia, spinal surgery*

Introduction

Perioperative hypothermia is associated with morbidity in general surgical patients (1). Adverse consequences of even mild hypothermia include morbid myocardial events (2), coagulopathy (3), increased transfusion requirements (4), surgical wound infection and prolonged hospitalisation (5, 6), prolonged recovery (7) and thermal discomfort (8).

Perioperative hypothermia is common and results from anaesthetic-induced inhibition of thermoregulatory control, core-to-peripheral heat redistribution and the cold operating environment. Thus, preventive measures to reduce perioperative hypothermia with active warming devices have become standard practice unless hypothermia is otherwise indicated. The most common perioperative warming system is convective warming with forced-air currents or air streaming over the patient (9, 10). Forced-air warming systems have consistently been shown to maintain normothermia, even during major operations (11). However, in major posterior approach spinal surgery, thermoregulation of patients can be challenging. A large area of the anterior body surface of these patients is exposed to the cold environment of the operating room, which can lead to major heat loss. Forced-air warming blankets have been used to warm only the upper posterior part of the patients' body or the lower limbs. Resistive heating systems provide alternative types of warming blankets as they can selectively heat up different body segments simultaneously. Resistive heating systems have comparable efficacy to forced-air warming systems in maintaining core temperature during major open abdominal surgery (12), total hip replacement surgery (13) and in heat transfer and core re-warming rates (14). Unfortunately, in posterior approach spinal surgery, the resistive heating system can warm only the upper posterior part of the body and the lower limbs. The anterior body surface area is still exposed to the cold operating room environment. Thus, there is a need for a warming blanket that can provide heat to this part of the body.

Recently, a new full-body forced-air warming blanket was developed, which is placed under the body of the patient undergoing spinal operation in the prone position on a Jackson table. This blanket covers the anterior body surface area and the lower limbs. It transfers heat to the available anterior skin surface, which is normally exposed to the cold environment.

Therefore, in this prospective, randomised study, our aim was to compare the efficacy of this new under-body forced-air warming blanket with that of resistive heating blanket in preventing hypothermia in patients undergoing major posterior approach spinal surgery.

Methods

With approval from the Ethics Committee of University Malaya Medical Centre and with written, informed consent, we studied 60 patients undergoing elective spinal surgery. All patients were American Society of Anesthesiologists (ASA) classification I or II, aged 12-80 years old, undergoing spinal surgery on a Jackson table in the prone position with operation duration of a minimum of two hours. Patients with preoperative pyrexia, evidence of current infection, thyroid disease, recent head injury with disturbance of autonomic functions or use of vasoactive drugs, were excluded from the study.

Participating patients were randomly assigned to:

1. Forced-air warming group: full under-body forced-air warming blanket that covers almost the whole ventral surface of the patient's body; face, anterior trunk, both upper limbs and lower limb placed underneath the body (Figure 1) with controller set to high temperature (42°C) (Bair Hugger ~ Arizant Healthcare Inc., USA) or
2. Resistive heating group: three pieces of carbon-fibre resistive heating blanket; one large upper body blanket that covers upper back and both upper limbs and two separate 'lower limbs' blankets that wrap up the lower limbs (Figure 2). The temperature of these blankets was set to 42°C (Thermamed SmartCare OP system).

Figure 1: Picture showing the application of resistive heating blanket on patient during spinal surgery.

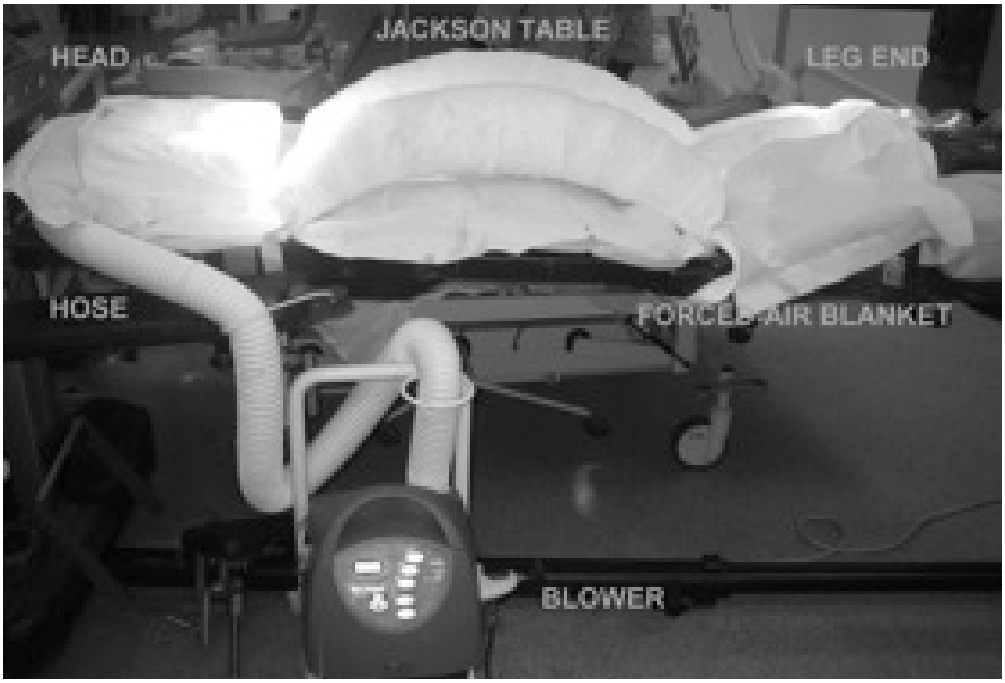

Figure 2: Picture showing the application of forced-air warming blanket on Jackson table during spinal surgery.

Randomisation was based on computer-generated codes sealed in numbered opaque envelopes and opened upon arrival of the patients in the operating room.

A thin layered cotton wool was used to wrap around the four limbs of all patients as soon as they arrived at the reception area. General anaesthesia was induced with fentanyl 1-2 µg/kg and propofol 2 mg/kg, followed by atracurium 0.5 mg/kg or rocuronium 0.6-0.9 mg/kg to facilitate tracheal intubation, and was then maintained with sevoflurane/O_2/air. All patients were mechanically ventilated to maintain end tidal CO_2 with partial pressure between 33 - 36 mmHg.

A nasopharyngeal temperature probe was then inserted into the nostril with a marking of 5 cm at the patient's nose. A rectal temperature probe was inserted into the rectum with the surface marking at 8 cm. A skin temperature probe was placed beneath one of the axilla of the patient with both arms in a diver's position. All temperatures were measured with thermocouples, with measurements starting at induction and continued throughout the surgery at 20-min intervals. Ambient temperature was measured with a thermocouple positioned at the level of the patient, well away from any heat-producing equipment. Skin temperature was measured at the axilla. Core temperature was measured at the nasopharynx and the rectum and was presented as an average value of two readings at any one time. Both core and skin temperatures were measured as a function of intraoperative time, with induction of general anaesthesia as time lapse of zero.

The operating room had controlled laminar air flow with room temperature set at 20°C and relative humidity of about 40%. All intravenous fluids were warmed in a fluid warming device (HOTLINE Level 1 set at 41°C). Patients were administered fluid according to this regime-maintenance of fluid with crystalloids at 7 ml/kg/h and replacement of blood loss with colloid at ratio of 1:1. The transfusion trigger was a haemoglobin level of less than 8 g/dl.

Demographic, morphometric and clinical characteristics of participants and duration of surgery were recorded. Blood pressure, heart rate, oxygen saturation and end tidal carbon dioxide were recorded at 20-min intervals.

To detect a clinically significant difference equal to 0.3°C in final mean core temperature, we prospectively calculated that 30 patients were required in each group, assuming a standard deviation of 0.4 °C (α=0.05; β=0.2) (12).

The distribution of data was evaluated with the Kolmogorov-Smirnov test. All intraoperative measurements were averaged over time at each time point among patients in each treatment group. Differences among groups were compared using the independent t-test for variables. P value ≤0.05 was considered statistically significant. Continuous variables were presented as mean ± standard deviation unless otherwise indicated.

Results

The characteristics and clinical details of patients in each group are shown in Table 1. No significant difference was found between groups. No evidence of thermal injury was observed related to any of the warming devices.

Table 1: Patient characteristics

	Forced-air warming blanket	Resistive heating blanket
Age (years)	35±17	44±18
Gender (Male / Female)	8 / 22	7 / 23
Weight (kg)	54.7	57.6
Body mass index (kg/m2)	22	23
Operation duration (min)	231±75	226.67 ±87
Mean Blood Pressure(mmHg)	72	75
Heart Rate (/min)	78	72
Oxygen saturation (%)	99	99
End tidal CO_2 (mmHg)	35	34

* Characteristics and clinical details of patients warmed with either forced-air warming blanket or resistive heating blanket during spinal operation. Values are mean or mean ± SD.

The baseline core temperatures in both groups were similar and did not significantly differ- 36.36± 0.38°C for forced-air group and 36.27 ± 0.46°C for resistive heating group. Patients in both groups showed a decrease in core temperature post-induction of anaesthesia. In the first hour, in the forced-air group, the core temperature decreased by 0.41°C from the baseline, while in resistive heating group the temperature dropped by 0.59°C from the baseline. This difference was not statistically significant (P< 0.05).

Core temperature increased at 100 minute post-induction until the end of the surgery in both groups. At the end of surgery, the core temperature in the forced-air group had increased by 0.08°C from the baseline, whereas, in the resistive heating group, the core temperature had decreased by 0.40°C from baseline (P< 0.05). (Figure 3)

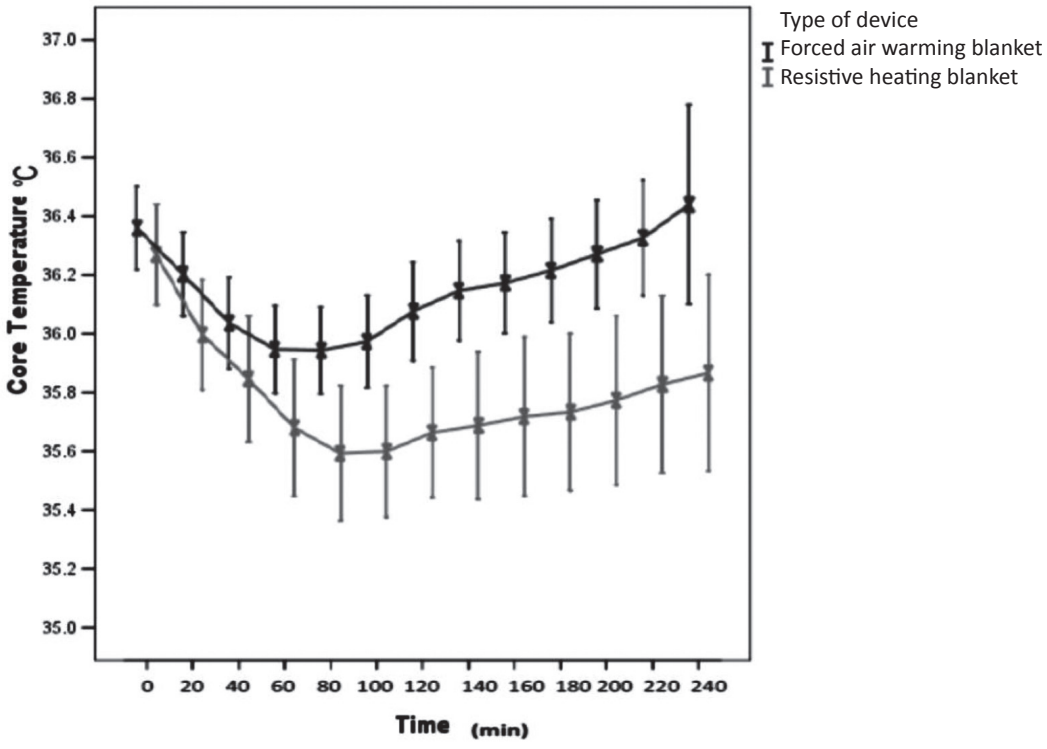

Figure 3: Core temperature in patients warmed with forced-air warming blanket or resistive heating device during spinal surgery at different time points. Values are mean ± confidence interval 95%.

Discussion

The forced-air blanket uses a warming system that depends on blower strength, air temperature and surface area covered to provide heat. The temperature of the blanket is controlled by a computer, which can be set between 32-42°C. The disposable blanket is designed to cover the ventral body area of patients on the spinal surgery bed. It is soft and radiolucent, thus accommodating intraoperative imaging.

The resistive heating blanket (Thermamed SmartCare OP system) is a non-disposable blanket with semi-conductive carbon fibre and low-voltage direct current to provide heat. Its heating ability is controlled by a computer that sets the temperature between 37-42°C. This blanket can independently warm several fields simultaneously. The blanket is made of semi-conductive carbon fibre and is therefore not radiolucent.

A thin layered cotton wool was used to wrap around the four limbs of all patients as soon as they arrived at the reception area in order to reduce the initial heat redistribution post-induction. The other reason for doing this is to avoid thermal injury due to direct contact of heat source, as a significant rise in temperature will be directly transferred to the patient with minimal dissipation. Burns have been reported with all types of heating blankets and are related to direct thermal injury (15) or due to device malfunction and inappropriate operation.

We have demonstrated that the efficacy of the full under-body forced-air warming blanket is superior over that of the resistive heating blanket in preventing hypothermia during major spinal operations lasting more than 2 hour. The superior efficacy of the forced-air blanket is probably due to the fact that it covered almost the entire ventral surface of the patients' body, whereas with the resistive blanket most of the anterior surface of patients' body was exposed directly to the cold ambient operation theatre temperature throughout surgery. The efficacy of active clinical warming devices is proportional to the available skin surface (16) and the design of the blanket (17).

A possible limitation of this study is that we did not analyse the blood loss at the end of surgery. However, we do not think our results are affected by this as we had a standardised regime to administer fluid for maintenance and replacement of any fluid and blood loss. In addition, all fluids administered to patients were warmed up to the point of entry into patients with fluid warming devices. Other studies also did not take into account the blood loss in their patients (18, 19).

Our results are inconsistent with those of several previous studies that showed comparable efficacy of forced-air warming, and resistive heating blankets in maintaining normothermia during open abdominal surgery (13) and laparoscopic cholecystectomy (18). Negishi *et al* (12) studied only eight patients in each treatment group undergoing open abdominal surgery with a forced-air warming blanket used to cover both lower limbs and resistive heating blanket used to cover one arm, chest and both legs. Matsuzaki *et al* (18) also studied eight patients in each group of patients undergoing laparoscopic cholecystectomy, with a forced-air warming blanket covering the upper body in the first group and a resistive heating blanket covering arms, chest and both legs in the second group. Thus, in these studies a larger surface was covered by resistive heating blankets than in our study.

We used the average of nasopharyngeal and rectal temperature as a measurement of core temperature. In other studies (13, 18), tympanic membrane temperature was used as core temperature. We decided to use the nasopharyngeal temperature as it has been shown to correlate well with the temperature of the thalamus, which is the site for thermoregulation. Rectal temperature was also measured, as it was shown to measure core temperature accurately and precisely (20). Therefore, by deriving core temperature from the average of these two measurements, it will best reflect core temperature of the patient. In addition, inserting an aural temperature probe into the tympanic membrane might damage the tympanic membrane, especially if it is inserted after general anaesthesia (21).

In this study, we showed that the full under-body forced-air warming blanket is able to keep the core temperature above the baseline to the end of surgery, whereas the core temperature at the end of surgery with the resistive heating blanket fell below baseline. As forced-air blankets are disposable, they can be more costly than resistive heating blankets. However, due to their efficacy and the importance of preventing hypothermia intra-operatively, we recommend that the full under-body forced-air warming blanket should be used in spinal operations.

Conclusion

This prospective and randomised study demonstrated the greater efficacy of the newly developed full under-body forced-air warming blanket over that of resistive heating blankets in preventing hypothermia in patients undergoing major posterior approach spinal surgery. Our study showed a blanket that covered a larger ventral body surface area was better in preventing intra-operative hypothermia than a blanket that covered a smaller surface area. Thus, in future, various designs of warming blanket should be developed to cater for different types of surgery.

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ALOE EMODIN ENHANCES TAMOXIFEN CYTOTOXICITY EFFECT ON ERα-POSITIVE BREAST CANCER CELLS, MCF-7, THROUGH DOWNREGULATION OF MEK1 AND MEK2

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ABSTRACT

The positive response to tamoxifen in ERα-positive breast cancer patients is usually of a short duration as many of the patients eventually develop resistance. Our preliminary results show that aloe emodin extracted from the leaves of the *Aloe barbadensis* Miller demonstrated a cytotoxicity that is selective to ERα-positive breast cancer cells (MCF-7), but not to ERα-negative breast cancer cells (MDA-MB-231) and to the control cells (MCF-10A). The objective of this study was to test the hypothesis that aloe emodin may enhance the response of MCF-7 cells to treatment with tamoxifen. MCF-7 cells were treated with aloe emodin alone, tamoxifen alone or a combination of emodin and tamoxifen, at their respective IC_{50} concentrations and at different time points of 24 hours, 48 hours and 72 hours. The respective IC_{50s} were the concentrations of aloe emodin and tamoxifen required to achieve 50% inhibition of the cells in the study. Cell viability and apoptosis were determined using trypan blue exclusion and DNA fragmentation assays, respectively. The involvement of RAS/MEKs/ERKs genes of MAPK signalling pathways with aloe emodin was determined using QuantiGene 2.0 Plex assay. Data was evaluated using the one-way ANOVA test. Our findings showed that aloe emodin enhanced the cytotoxicity of tamoxifen on MCF-7 cells through apoptosis by downregulation of MEK1/2 genes. Our research may provide a rational basis for further *in vivo* studies to verify the efficacy of a combination of aloe emodin and tamoxifen on the viability of ERα-positive-breast cancer cells.

Keywords: *Aloe emodin, tamoxifen, MAPK, MCF-7.*

Introduction

Breast cancer is the most common cancer in women, with the highest prevalence among Asians including Malaysians (1, 2). A Malaysian cohort study from the year 2000 to 2005 showed that the overall 5-year survival rate of breast cancer patients among Malaysian women was lower compared to survival rates in developed nations (2). Nearly two thirds of all breast cancer patients expressed estrogen and progesterone receptors, responding with growth to these hormones (3). Estrogen stimulated the proliferation of estrogen receptor α (ERα)-positive breast cancer cells through the activation of the extracellular signal-regulated kinase (ERK) proliferation pathway (4). Tamoxifen is a selective estrogen receptor modulator (SERM) and directly competes with estrogen for receptor binding (5). It is an endocrine therapy of choice for treatment of advanced ERα-positive breast cancer patients as it demonstrates a benefit when used alone and in combination with other chemotherapeutic agents (5). It reduces tumour recurrence, prolonging survival when administered as a post-operative adjuvant therapy in stages I and II of the disease (5, 6).

Deregulation of ERK pathway is seen in approximately one third of all human cancers (6). The ERK is one of three subfamilies of the mitogen-activated protein kinases (MAPKs). The others are c-Jun N-terminal kinase (JNK) and

p38 kinase. The MAPKs pathways control the essential homeostatic balance between proliferation and death of cells in the fundamental development and maintenance of cellular processes (6). The ERKs and JNKs control cell proliferation and apoptosis respectively, whilst p38 is activated by inflammatory agents (6). The ERK pathway consisting of RAS/RAF/MEK/ERK is critical in transmitting proliferative extracellular signals such as growth factors or mitogens generated by cell surface receptors, to regulate gene expression and prevent apoptosis (6, 7). Abnormal ERK signalling is prevalent in most cancers as a consequence of increased expression or mutation of its upstream components (8). It may lead to uncontrolled cell proliferation, resistance to apoptosis, and resistance to chemotherapy, radiotherapy and targeted therapies (8). The activation of RAF/MEK/ERK pathway leads to phosphorylation of pro-apoptotic Bad, thus allowing anti-apoptotic Bcl-2 to form homodimers and prevent apoptosis (9). It has been shown that the activation of RAF/MEK/ERK cascade inactivates Caspase-9 by phosphorylation thus blocking apoptosis (10). Increased expression of RAS/RAF/MEK/ERK pathway is associated with a poor prognosis in ERα-breast cancer patients (4, 8). Activation of RAS pathway increases the sensitivity of ERα to low concentrations of estrogen and reduces tamoxifen efficacy (11, 12). Over expression of ERK1/2 is frequently seen in association with advanced stage of ovarian (13) and prostatic (14) cancers and their poor prognosis. There is a continuing effort in developing new strategies to enhance and prolong the efficacy of tamoxifen therapy. Aloe emodin (1, 8-dihydroxy-3-hydroxymethyl-anthraquinone) is found abundantly in *Aloe barbadensis* Miller leaves. It is phytoestrogenic due to its ability to limit the proliferation of ERα-positive breast cancer cells by downregulating ERα, thus suppressing ERα transcriptional activation. It has no effect on ERα-negative cells proliferation (15). As analogues, synthetic estrogen-like compounds such as tamoxifen competes for estrogen binding sites of ERα and therefore inhibits the growth of ERα-dependent breast cancer cells (12). These analogues exhibit side effects that increase the risk of cancer development due to unselective estrogenic action (12). The estrogenic potency of natural phytoestrogens is generally lower than synthetic estrogens and they have fewer side effects (16). Aloe emodin and its isomer, emodin (1,3,8-trihydroxy-6-methylanthraquinone), also enhance the inhibitory effect of chemotherapeutic agents such as cisplatin, doxorubicin, 5-fluorouracil and tyrosine kinase inhibitor (STI 571) on Merkel cell carcinoma (MCC) and prostate cancer cells (DU-145) especially at low concentrations of drugs used (17,18). Aloe emodin is slightly more potent compared to emodin (16). These findings provide the evidence to suggest that aloe emodin is a suitable candidate for use in combination with chemotherapeutic agents such as tamoxifen in the management of ERα-positive breast cancer patients. Further investigation is required to determine the underlying signalling pathways of aloe emodin-induced cell death in MCF-7 cells.

Methods

Treatments and reagents

Aloe emodin (≥95% HPLC) and tamoxifen were each dissolved in dimethyl sulfoxide (DMSO) to prepare primary stocks of 50mM. Each final working solution of aloe emodin and tamoxifen were diluted in culture media so that the final concentration of DMSO in cell culture was <0.1% (GIBCO Invitrogen, USA). Aloe emodin, tamoxifen and DMSO were purchased from Sigma Chemical Co., USA. Disposable sterile consumables (Brand Axygen, USA) and chemicals used were of tissue culture grade.

Cell lines and culture

Both ERα-positive and ERα-negative breast cancer cells, MCF-7 and MDA-MB-231, respectively (American Type Culture Collection, USA) were cultured in complete RPMI 1640 media supplemented with 10% fetal calf serum and 1% of penicillin and streptomycin (GIBCO Invitrogen, USA). ERα-negative non-transformed breast cells, MCF-10A (American Type Culture Collection, USA) were maintained in complete DMEM high glucose media supplemented with 5% horse serum, 20ng/ml of EGF, 0.5mg/ml of hydrocortisone, 10μg/ml insulin and 1% of penicillin and streptomycin (GIBCO Invitrogen, USA). All cells were maintained as a monolayer up to 80% confluence in humidified atmosphere of 5% CO_2, at 37°C in T25 and T75 flasks (Orange Scientific, Belgium). All cell culture experiments were performed under sterile condition in tissue culture hood.

Cell viability

Cell viability was performed using trypan blue exclusion assay (Sigma Chemical Co., USA). Cells were seeded at optimized 4 x 10^5 in a 6-well plate (Orange Scientific, Belgium) and treated with aloe emodin, tamoxifen and the combination of both at their respective IC_{50} concentrations obtained from WST-1 proliferation assay (Amin *et al*., 2013) for 24 hours, 48 hours and 72 hours in 5% CO_2 at 37°C. Tamoxifen was used as positive control. Cells cultured in complete media with 0.1% of DMSO were used as negative control. Cell morphology was observed under an inverted light microscope (Olympus, Japan) at 100X magnification. An equal volume of cell suspension and 0.4% trypan blue dye were mixed thoroughly and allowed to stand for 5 minutes. From the sample mixture, 10mL was added into the chamber port on the Countess Cell counting chamber slide (Invitrogen, Canada). Slides were inserted into the port and counted. A viable cell appeared as a bright centre with dark edges, while a dead cell was blue and without any bright centre (19).

Cell apoptosis

Cell apoptosis was determined using morphological evaluation with acridine orange (AO; Sigma Chemical Company, USA) and propidium iodide (PI; Sigma Chemical Company USA) dual staining technique. A total of 4 x 10^5

cells were seeded in a 6-well plate (Orange Scientific, Belgium) and treated with aloe emodin, tamoxifen and the combination of both at their respective IC_{50} concentrations for 48 hours and 72 hours in 5% CO_2 at 37°C. Tamoxifen and untreated cells were used as positive and negative controls, respectively. After staining with 1mg/mL AO and 1mg/mL PI for 30 minutes, the fluorescent images were captured using fluorescence microscope (Olympus, USA) at 200X magnification. A total of ≥200 to 300 cells on four sub-grids of a haemocytometer was counted to determine the percentages of viable, apoptotic and necrotic cells.

Gene expression

The involvements of RAS, MEK1/2 and ERK1/2 in the underlying apoptotic signalling pathways induced by aloe emodin, tamoxifen and the combination of both in MCF-7 were determined using QuantiGene 2.0 multiplex assay (Affymetrix, USA). Ubiquitin C (UBC) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) were used as housekeeping genes (Affymetrix, USA). A total of 1 x 10^6 cells were treated with the same treatment groups as above, for up to 72 hours. Cell pellets were resuspended and kept at -20°C before use. The assay was further conducted following the protocol, based on the series of hybridization methods that captured target RNA in the samples. They analyzed the expression of RNA through amplification of signal that can be detected by flow cytometry (I-DNA Biotechnology (M) Sdn. Bhd., Malaysia).

Statistical analysis

The differences between groups were evaluated using one-way ANOVA. Each experiment was repeated three times independently and in triplicates. Data obtained was expressed as the mean ± standard deviation, n=3. Significance was set up at $p<0.05$.

Results

Effects of aloe emodin and tamoxifen on the viability of MCF-7

Previously, our group had determined the respective IC_{50} of aloe emodin and tamoxifen using WST-1 proliferation assay. Aloe emodin selectively inhibited the proliferation of ERα-positive breast cancer MCF-7 cells with IC_{50} of 80μM at 72 hours. No IC_{50} was obtained for ERα-negative breast cancer MDA-MB-231 and ERα-negative non-transformed breast MCF-10A cells. In contrast, tamoxifen was non-selective to all three cells with IC_{50} of 27μM, 19μM and 38μM, respectively (20). We further investigated the effect of aloe emodin and tamoxifen on MCF-7 cell viability using trypan blue exclusion test to reconfirm these findings. The representative microscopic images of aloe emodin, tamoxifen and combined treatments groups on MCF-7 cell viability compared to untreated cells were shown in **Figure 1A**. Aloe emodin and tamoxifen inhibited the viability of MCF-7 cells by 32.00±1.19% to 43.18±3.21% ($p<0.05$, n=3) and 14.78±2.60% to 16.49±1.61% ($p<0.05$, n=3), respectively compared to untreated cells at 24 hours and up to 72 hours. Aloe emodin was a more potent inhibitor than tamoxifen ($p<0.05$, n=3). Tamoxifen cytotoxicity on MCF-7 cells was significantly enhanced by aloe emodin with 22.05±1.20%, 37.50±2.23% and 36.93±2.19% ($p<0.05$, n=3) increment at all treatment time points (24, 48 and 72 hours) **(Figure 1B)**. Aloe emodin and tamoxifen respective IC_{50} concentrations were also used in apoptosis and gene expression assays.

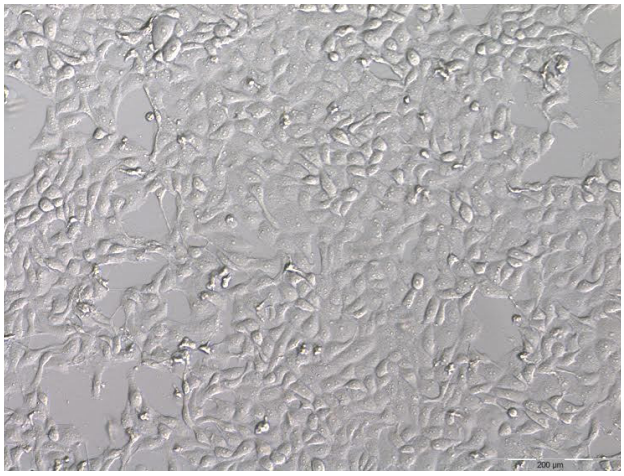

Figure 1A: Untreated image of MCF-7 cells

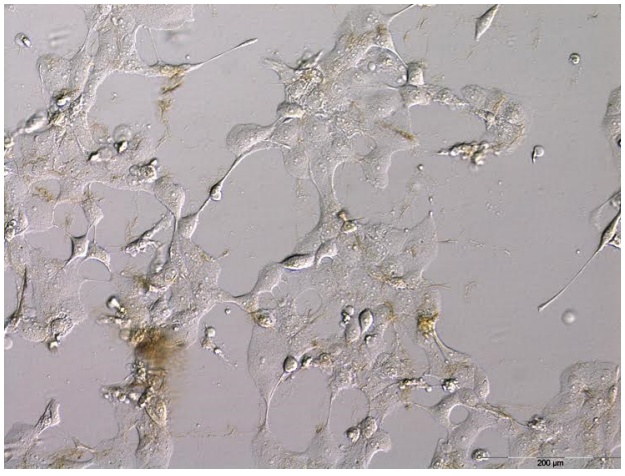

Figure 1A: Aloe emodin treated image

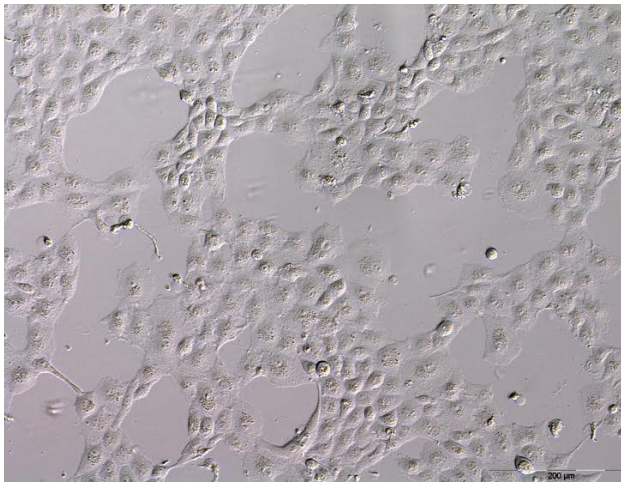

Figure 1A: Tamoxifen treated image

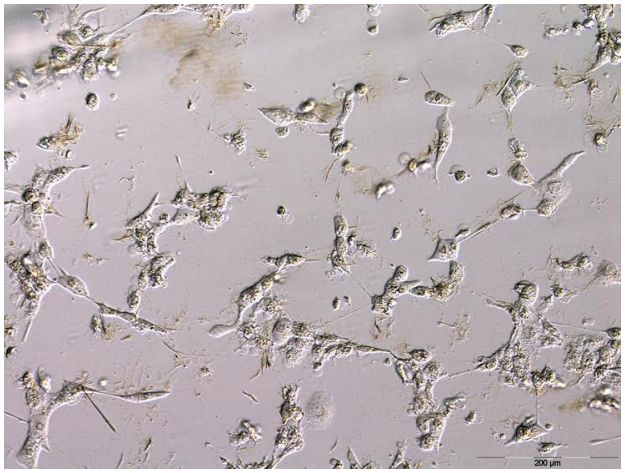

Figure 1A: Aloe emodin and tamoxifen treated image.

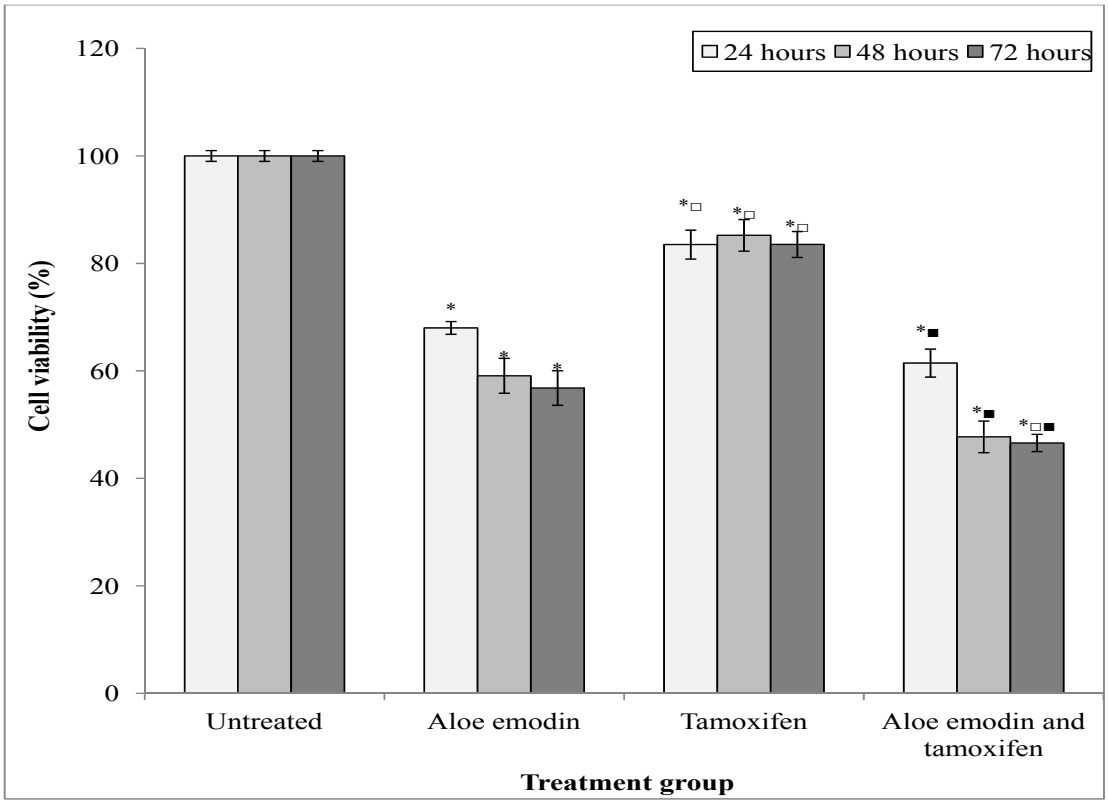

Figure 1B: Overall percentage of cell viability ± SD obtained from three sets of experiments and in triplicates (n=3) in a time-dependent manner

Figure 1: Cell viability effect of aloe emodin, tamoxifen and combination of both agents on MCF-7. The representative microscopic images of all three treatment groups compared to untreated cells **(A)**. 4 x 10^5 cells were incubated with aloe emodin, tamoxifen and combination of both agents at respective IC_{50} up to 72 hours in 5% CO_2 at 37°C. Cell morphology was observed under an inverted light microscope. The overall percentage of cell viability ± SD obtained from three sets of experiments and in triplicates (n=3) in a time-dependent manner is presented in bar graph **(B)**. Aloe emodin is more potent as an inhibitory agent of MCF-7 viability compared to tamoxifen. Furthermore, tamoxifen cytotoxicity is enhanced by aloe emodin at all treatment time. *Significant as compared to untreated cells at $p<0.05$; □Significant as compared between aloe emodin with tamoxifen group at $p<0.05$; ■Significant as compared between the combined treatment of aloe emodin and tamoxifen with tamoxifen alone in MCF-7 at $p<0.05$.

Effects of aloe emodin and tamoxifen on MCF-7 cell apoptosis

The percentages of viable (V), early apoptotic (EA), late apoptotic (LA) and necrotic (N) cells determined using AO and PI dual staining were represented by the images above **(Figure 2A)**. A viable (V) cell appeared as green with intact nucleus. An early apoptotic (EA) cell was bright green from intercalation of both dyes in the nucleus. A late apoptotic (LA) cell was red/orange with the generation of the highest PI intensity emission. A necrotic (N) cell was orange with disrupted cell membrane. The results were represented in **Figure 2B**.The presence of early and late apoptotic MCF-7 cells were seen after 48 hours of aloe emodin (16.33±3.12% and 10.55±2.31%; $p<0.05$, n=3) and tamoxifen (18.55±2.51% and 11.50±2.61%; $p<0.05$, n=3) treatment compared to untreated cells. Increased apoptosis was seen in the combined treatment of aloe emodin with tamoxifen (25.23±3.10% of early and 15.21±3.23% of late apoptosis; $p<0.05$, n=3) compared to untreated cells **(Figure 2B(a))**. More cells underwent late apoptosis compared to early apoptosis after 72 hours of treatment. Higher apoptotic effect was also seen in the combined treatment of aloe emodin with tamoxifen (25.32±2.50% of early and 50.22±2.51% of late apoptosis; $p<0.05$, n=3) compared to aloe emodin (15.52±3.25% of early and 35.31±2.23% of late apoptosis; $p<0.05$, n=3) and tamoxifen alone (13.22±2.01% of early and 30.25±3.22% of late apoptosis; $p<0.05$, n=3) **(Figure 2B(b))**.

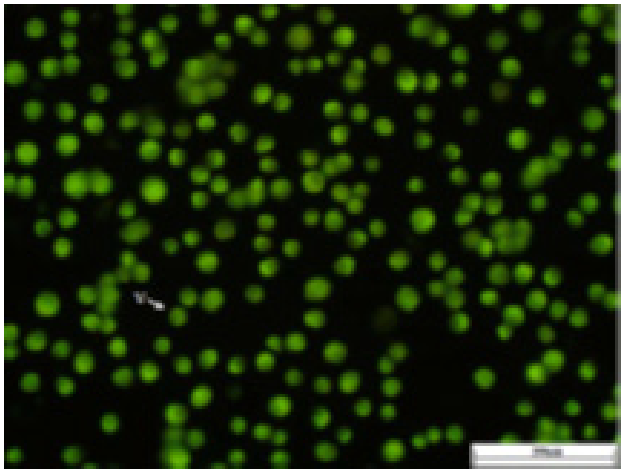

Figure 2A(a): Untreated MCF-7 cells image (48 hours)

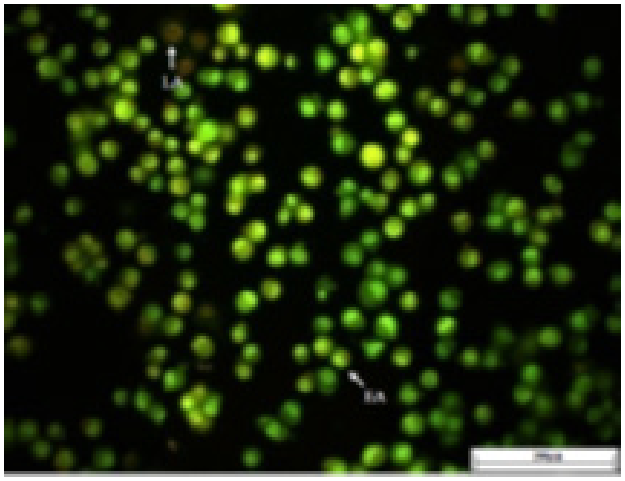

Figure 2A(a): Aloe emodin treated image (48 hours)

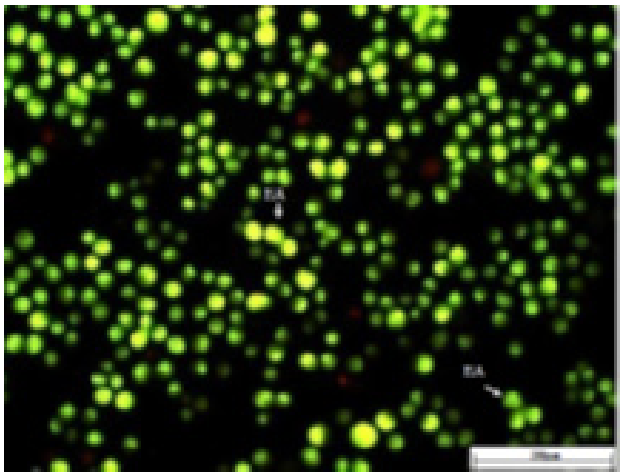

Figure 2A(a): Tamoxifen treated image (48 hours)

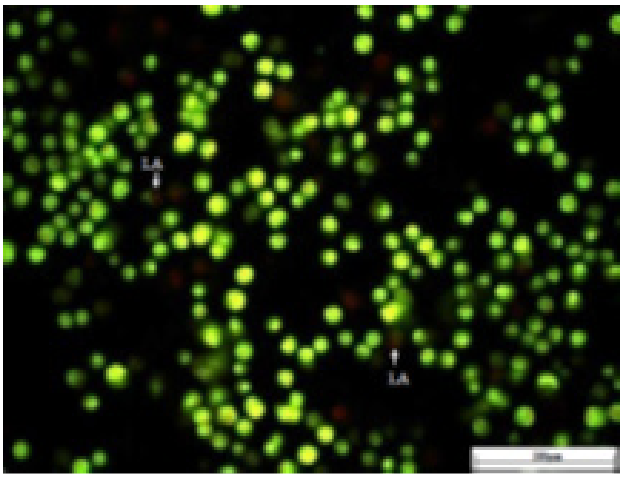

Figure 2A(a): Aloe emodin and tamoxifen treated image (48 hours)

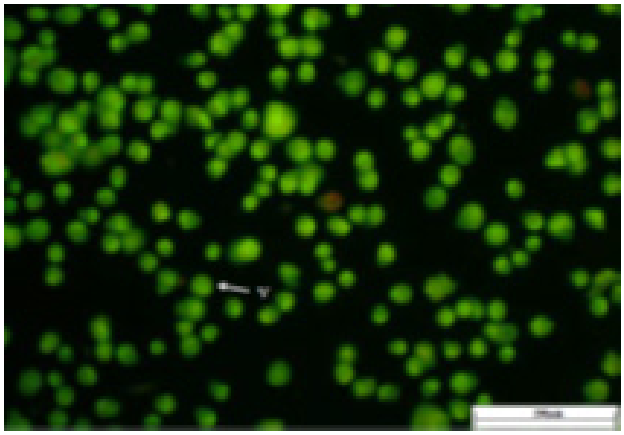

Figure 2A(b): Untreated MCF-7 cells image (72 hours)

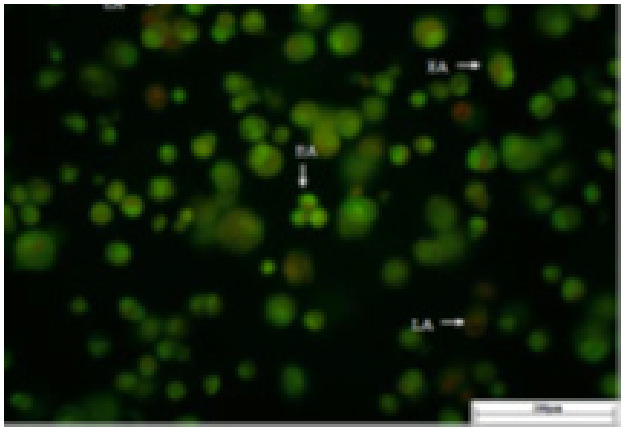

Figure 2A(b): Aloe emodin treated image (72 hours)

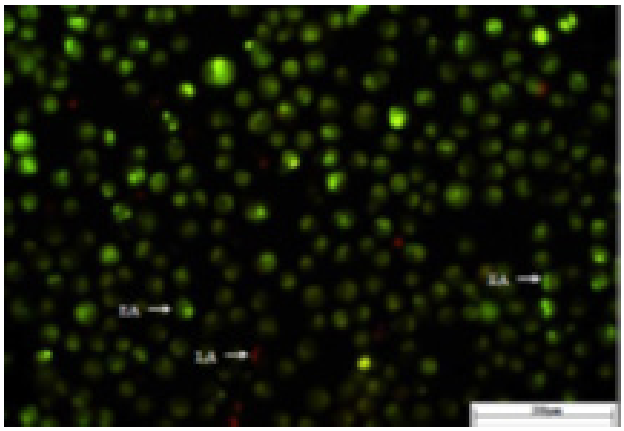

Figure 2A(b): Tamoxifen treated image (72 hours)

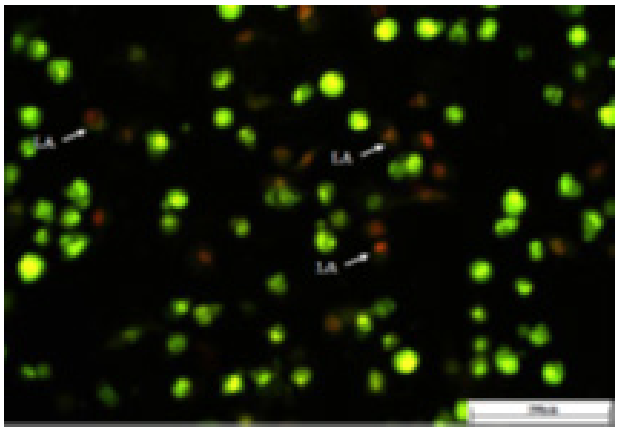

Figure 2A(b): Aloe emodin and tamoxifen treated image (72 hours)

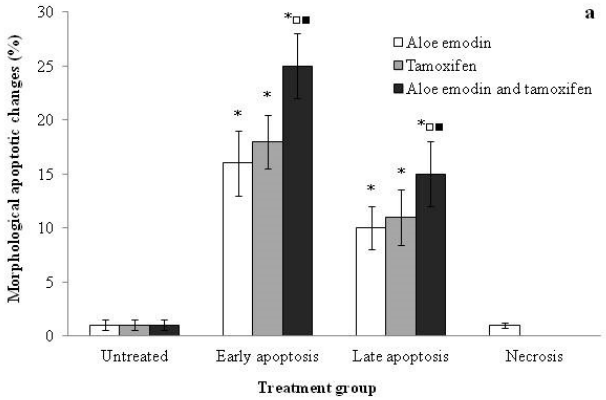

Figure 2B(a):

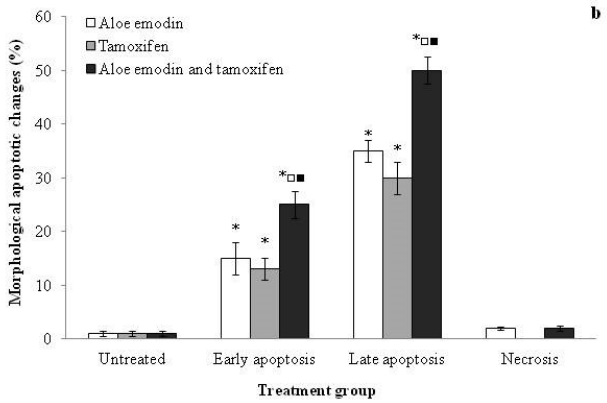

Figure 2B(b):

Figure 2: Apoptotic morphological changes of MCF-7 cells after treatment with aloe emodin, tamoxifen and combination of both agents. 4 x 10^5 cells were treated with aloe emodin, tamoxifen and combination of both agents at respective IC_{50} up to 72 hours in 5% CO_2 at 37°C. Apoptotic morphological changes were observed by applying AO and PI dual staining. Cells were identified as viable (V), early apoptosis (EA), late apoptosis (LA) and necrosis (N) after 48 hours and 72 hours of treatment represented by **Figure 2A(a)** and **2A(b)**, respectively**.** The percentage of these changes obtained from all three sets of experiments and in triplicate (n=3) at different times (48 hours and 72 hours) was represented in the **Figure 2B(a)** and **Figure 2B(b)**, respectively. At 48 hours, higher apoptosis was seen in aloe emodin and tamoxifen combined treatment in MCF-7 cells compared to aloe emodin and tamoxifen alone **(Figure 2B(a))**. Similar pattern was seen at 72 hours (**(Figure 2B(b))**. *Significant as compared to untreated cells at $p<0.05$; □Significant as compared between the combined treatment of aloe emodin and tamoxifen with aloe emodin alone at $p<0.05$; ▣Significant as compared between the combined treatment of aloe emodin and tamoxifen with tamoxifen alone at $p<0.05$.

Effects of aloe emodin and tamoxifen on the RAS, MEK1/2 and ERK1/2 signalling of MCF-7

The effect of aloe emodin and tamoxifen-combined treatment on the expression of RAS, MEK1/2 and ERK1/2 genes in the MAPK proliferation pathway of MCF-7 was determined. UBC housekeeping gene was used to normalize the expression of target genes. Similar pattern was seen using HPRT gene. All the target genes were downregulated by the respective aloe emodin and tamoxifen treatment in MCF-7 **(Figure 3).** Synergistic effect was seen in MEK1/2 genes **(Figure 3B)**.

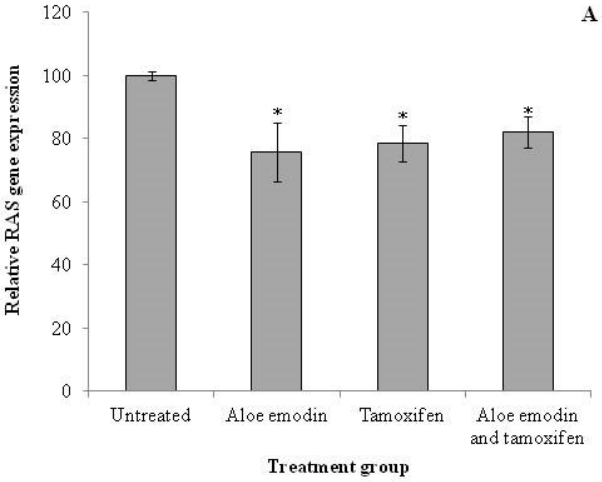

Figure 3A

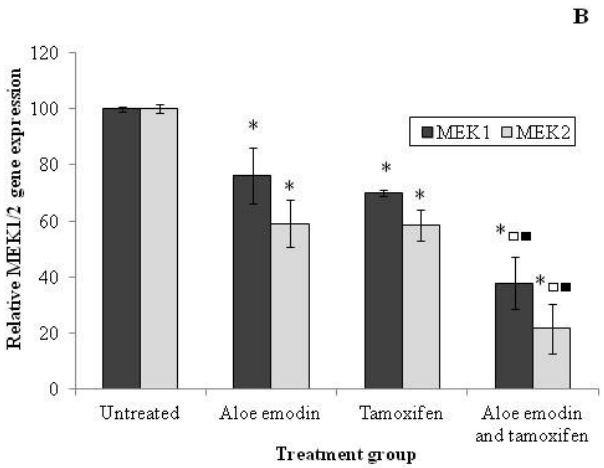

Figure 3B

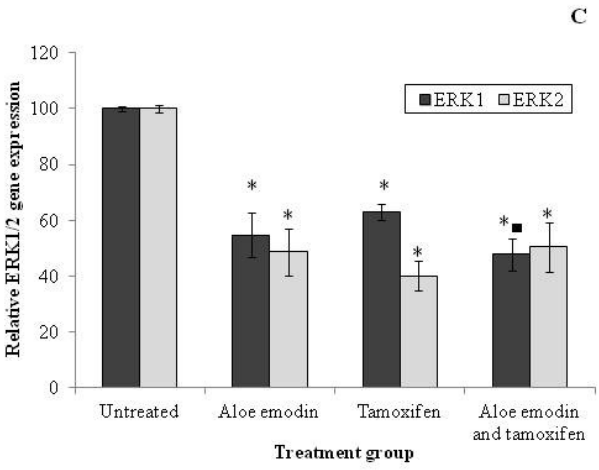

Figure 3C

Figure 3: RAS (A), MEK1/2 (B) and ERK1/2 (C) gene expression of MCF-7 cells after treatment with aloe emodin, tamoxifen and combination of both agents. 1 x 106 cells were treated aloe emodin, tamoxifen and combination of both agents at respective IC50 up to 72 hours in 5% CO2 at 37oC. The assay was conducted following the QuantiGene 2.0 multiplex assay. The mRNA level of all target genes was expressed as % normalized to UBC housekeeping gene ± SD. The experiments were duplicated and repeated thrice (n=3). Similar pattern was seen using HPRT as housekeeping gene. Higher downregulation of MEK1/2 genes expression were seen in the combined treatment compared to aloe emodin and tamoxifen alone, respectively ($p<0.05$). *Significant as compared to untreated cells at $p<0.05$; □Significant as compared between the combined treatment of aloe emodin and tamoxifen with aloe emodin alone at $p<0.05$; Significant as compared between the combined treatment of aloe emodin and tamoxifen with tamoxifen alone at $p<0.05$.

Discussion

Aloe emodin, a hydroxyanthraquinone from *Aloe barbadensis* Miller leaves, has been found to have anti-cancer effects on several cancer cell lines such as Merkel and prostate (17,18). It is non-toxic as demonstrated in an *in vivo* model (21). The effect of aloe-emodin on ERα-positive breast cancer cells is not well understood. In the previous study, we clearly demonstrated the selective anti cancer activity of aloe emodin on ERα-positive breast cancer cells, MCF-7 but not on ERα-negative breast cancer, MDA-MB-231 and ERα-negative non-transformed breast cells, MCF-10A (20). Unlike tamoxifen, aloe emodin was selectively cytotoxic for MCF-7 breast cancer cells (20). The inhibitory effect of Aloe emodin and tamoxifen on MCF-7 at IC_{50} of 80mM which had been published previously (20) was reconfirmed by trypan blue exclusion test. A combination treatment of aloe emodin with tamoxifen further inhibited the viability of MCF-7, reflecting an enhancement in the cytotoxicity effect significantly with $p<0.05$ **(Figure 1B)**. To examine the mechanism responsible for cell viability inhibition by both treatments, morphological apoptotic characteristics were evaluated. The findings suggested that cytotoxicity of aloe emodin and tamoxifen on MCF-7 cells was through apoptosis, not necrosis. An enhancement in apoptotic effect was seen in the combination treatment **(Figure 2A)** as represented by **Figure 2B**. Chen *et al* (22) showed a cytotoxicity effect using aloe emodin-loaded solid lipid nanoparticles (AE-SLNs) on MCF-7, but not on MCF-10A. AE-SLNs were prepared in an attempt to improve the anti-cancer efficacy of aloe emodin (22). It exhibited excellent cytotoxicity against MCF-7 but not on MDA-MB-453 cells (15)**.** Aloe emodin was selectively active against neuroectodermal tumour cells in both *in vitro* and *in vivo* models, and induced cell shrinkage, membrane blebbing and nuclear fragmentation; but not in human hematopoietic progenitors and normal fibroblasts (21). The hematopoietic cells were not affected at concentrations more than 100 times higher than those required to inhibit neuroectodermal tumour cell growth (21). Lymphoid cells (JURKAT and CCRF-CEM) and the most resistant of myeloid cells (KG-1a and K562) were responsive to aloe emodin through induction of apoptosis, but not in non-tumour cells (23). Similar effects were seen with its isomer, emodin (1,3,8-trihydroxy-6-methylanthraquinone) (23). Both compounds produced a synergistic effect when combined with etoposide and doxorubicin on both lymphoid cell lines (24). The combination of aloe emodin and radiation enhanced radiosensitivity and apoptosis of cervical cancer cells, HeLa, compared to treatment with aloe emodin or radiation alone (25). Co-treatment of emodin, with cisplatin significantly elevated ROS levels and enhanced the chemosensitivity in prostatic cancer DU-145 cells, compared with cisplatin alone. The combination therapy had little effect on normal human dermal fibroblasts (18). These evidences showed that aloe emodin and emodin had a potential role of enhancing the action of chemotherapy in cancer treatment. A significant regression was seen among patients with metastatic solid tumours. These tumours included lung cancer treated with cisplatin and etoposide, colorectal cancer on oxaliplatin and 5-fluorouracil; gastric cancer on 5-fluorouracil, and pancreatic cancer receiving gemcitabine. The patients were randomised to a group concomitantly treated with *Aloe arborescens* extracts (10mL thrice/daily orally) and a group on chemotherapy alone. An increment in the percentage of a 3-year survival rate was also noted (26). Further study to investigate the possible signalling pathways is required.

The MAPKs signalling pathway is a key intracellular cascade in the regulation of normal cell proliferation, cell cycle, survival, angiogenesis and migration. Alteration in the family components particularly the ERKs as survival protein cascades contribute to cancer and other human diseases (7). It has been the focus of interest in the development of pharmacologic inhibitors for the treatment of cancer (6-8). To elucidate whether the expressions of RAS, MEK1/2 and ERK1/2 are involved in aloe emodin-induced apoptosis in MCF-7 cells, we examined the expression of these genes using QuantiGene 2.0 multiplex techniques during aloe emodin-mediated apoptosis. Exposure of MCF-7 cells to aloe emodin at IC_{50} resulted in decreased RAS, MEK1/2 and ERK1/2 expressions after 72 hours of treatment, similar to tamoxifen. Combination of aloe emodin and tamoxifen significantly reduced the expression of MEK1/2 with $p<0.05$ **(Figure 3B)**. Aloe emodin also induced apoptosis in lung non-small carcinoma cells, H460, through inactivation of the ERK signalling pathway (27). Elevated levels of Rad51 expression seen in chemo or radioresistant lung carcinomas are decreased in non-small-cell lung cancer cells, H1703 and A549 by the synergistic effect of emodin (*Rheum palmatum L* root and rhizome extracts) with anti-tumor antibiotic mitomycin C through inactivation of ERK pathway (28). It enhanced the cytotoxicity effect of cisplatin in advanced non-small cell lung cancer cells, (NSCLC) through the downregulation of excision repair cross-complementation 1 (ERCC1), an enzyme crucial for the removal of adducts from genomic DNA, and inactivation of ERK survival pathway (29). Alizarin (1,2-dihydroxyanthraquinone), another anthraquinone from madder root extract inhibited osteosarcomas such as Saos-2, MG-63 and U-2 OS cells at lower doses compared to normal cells, demonstrating a selective activity towards malignant cells. It acted through the inhibition of ERK1/2 phosphorylation and S phase arrest (30).

The data obtained from this study is useful for future work on the apoptotic effects of aloe emodin at protein level.

Conclusion

Our findings are in line with published work which showed that natural anthraquinone selectively inhibited the proliferation of cancer cells at a lower dose compared to the normal cells, by regulating the ERK signalling pathway. We showed that aloe emodin enhanced tamoxifen cytotoxicity on ERα-positive breast cancer, MCF-7 cells, was mediated by MEK1/2 downregulation. These findings may provide a basis for the therapeutic usage of aloe emodin to enhance the efficacy of chemotherapy in ERα-positive breast cancer patients.

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Competing interests

The authors declare that they have no competing interests.

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ATTENDANCE AND INSTITUTIONAL FACILITIES OF LONG-TERM KIDNEY DONORS FOLLOW-UP

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ABSTRACT

Introduction:

Living donation is an important source for organs transplantation in Malaysia. This study aims to investigate the Malaysian living donors' follow-up attendance, their preferences on medical-institutional facilities, and the financial circumstances pertaining to the follow-up costs

Materials and Methods:

Primary data were collected through a survey of 80 living donors who made their donation at the University of Malaya Medical Center (UMMC) between 1991 and 2012.

Results:

Out total of 178 donors, only 111 were reachable and 80 of them participated in the survey (72%). The findings revealed that most of the donors (71.2%) attend the follow-up regularly. Nevertheless, donors seem to neglect the importance of follow-up as they consider themselves healthy (28.9%) or consider the follow-up as being troublesome (28.9%). Most donors (67.5%) are not in favour of being treated as patients, but prefer to be monitored under donor registry (88.8%) and getting their health service in special clinics for donors (80%). The majority of the donors fund the follow-up costs themselves (32.4%), while 25% of the donors' follow-up costs were funded by family members. Among those donors without income and those of low-income (84.8% of respondents), 60.3% believe that the follow-up costs should be borne by the government.

Conclusions:

Based on the findings, it is therefore suggested that the government provides all living donors with proper free health service through donor registry and donor clinics. Adequate care has to be given to the donors to pre-empt any unforeseen health complications due to the organ donation surgical procedures.

Keywords: *Living donors, follow-up, kidney donation, Malaysia*

Introduction

Thousands of patients around the world are dying every year due to kidney failure. The advancement in transplantation has given the world a new source of treatment for end-stage kidney failure (1). Nevertheless, the shortage of donated organ has severely impeded the number of transplantation in many countries, and Malaysia is no exception (2). For instance, on average, there had been only about 100 kidney transplantations performed yearly in Malaysia (3).

Kidneys can be donated by any living or deceased individual, yet the importance of living donation varies from one country to another. In countries with successful organ donation experience, such as Spain, Croatia and Malta, deceased donation forms the main pool of organ donation (4). This finding makes the source from living donation of lower importance (3). In Spain, for instance, the rates of organ donation were 35.12 per million population (PMP) for deceased and 8.59 PMP for living donation (3).

In Malaysia, living donation is of huge importance as a source of organ transplantation for two reasons. The first reason is the huge gap between the demand for organs and the low donation rate. In 2012, there were 28,590 dialysis patients, and although about half of them were registered on the waiting list for transplantation, there were only 94 transplantations, involving living and deceased donors, performed in that year (5). The second reason is that living donation outnumbers deceased donation by more than 300%, although both have been recording very low rates. In 2013, it was recorded that there was 1.87 PMP living donation compared to only 0.5 PMP for deceased donation (3).

Malaysia adheres to the Informed Consent system, in which those who are willing to donate their organs after death must register officially during their life time (6, 7). As per the official records, there were no "unrelated living donation" in the last two decades (8). In Malaysia, past experiences indicated that living donations has, thus far, been only within the family. This means living donors have only donated organs to genetically or emotionally connected recipients (8). The situation has remained the same as in the yesteryears even after the government has introduced clear guidelines and procedures for "unrelated living donations" in 2011 (9). To improve the rate of living donation, donors must be assured that their quality of life (QoL) after donation would not be compromised (10). They should also be helped to avoid any financial hardship as a consequence of donating their organ. The fear of medical or financial risks of organ donation could gravely lessen donation rates (11). A study on 133 potential donors revealed that 24% of them had not donated their organs due to their fear of facing financial hardship after donation (12). In another study, financial hardship was reported by about 23% of living donors after donating their organs (13).

One of the imperative factors to maintain donors' standard QoL is that they should attend post-donation follow-up sessions to monitor their health status (14). The importance of follow-up is stressed in the world health organization (WHO) principles of organ donation which states that "live donations are acceptable when the donor's informed and voluntary consent is obtained, when professional care of donors is ensured and follow-up is well organized" (15).

The Malaysian transplant policy requires living donors to go for follow-up (Clause 3.4), and that their welfare will be taken care of by the government (Clause 2.5) (16). However, the follow-up was left at donors' discretion and there is no institutionalized living donor registry to monitor their follow-up activities. In 2012, free secondary and tertiary medical facilities were only granted to donors who donated after 2012 (17). This means that the burden of follow-up costs is not borne by the government for those who donated before 2012.

Previous studies showed that living kidney donors do not suffer from any reduction in their QoL (18, 19), or an increase in mortality rate (20-22), compared to non-donors who are in the control group. However, the literature shows that a small proportion of living donors in the United States and adhere to their long-term follow-up (19, 23). In the case of Malaysia, an earlier study of 80 living kidney donors revealed that they have better QoL compared to 80 healthy individuals (24). However, to the best of our knowledge, there have been no studies which explore the follow-up status and wellbeing of the Malaysian living donors. Thus, this study aims to investigate the Malaysian living donors' follow-up attendance, their preferences on medical-institutional facilities, and the financial circumstances pertaining to the follow-up costs.

Methods

To achieve the objectives of the study, we examine the perspectives of a sample of living donors who donated their organs at the University Malaya Medical Centre (UMMC) between 1991 and 2012. The time frame of between 1991 and 2012 indicates that the donors involved in this study are those who did not have access to free follow-up medical facilities. Out of 178 donors, only 111 were reachable and 80 of them participated in the survey (72%). From the 31 reachable non-respondent donors, 11 stated that they were not free, 10 refused to participate, 5 were abroad, 2 were having their follow-ups in other hospitals, 2 had passed away and 1 was chronically ill.

A pilot-tested questionnaire was developed and two enumerators (one from UMMC and the other, an independent third party) were tasked to assist the donors to fill out the questionnaire.

First, donors were asked to indicate the frequency of attending the follow up sessions. They were given three options to choose from: 'regular', 'non-regular', and 'never' attend. Next, all respondents were asked to choose two reasons (out of six) as to the causes of their not attending the follow up sessions. The decision to give the option to choose two out of six reasons was based on the request by respondents captured during the pilot study. We understood from the pilot study that we may encounter low response rate. Therefore, the following reasons were developed based on hypothetical assumptions by the donors who attended regular and non-regular follow-up sessions. The donors were asked about their possible reasons if they have chosen not to attend the follow-up sessions. The six reasons were developed based on literatures and discussions with nephrologists, surgeons, and psychiatrists from UMMC. The reasons were as follows: (1) I am just as healthy as anybody I know; (2) the doctor did not advise me to do so; (3) I do not want to take the

trouble; (4) I do not want to be used as a research object; (5) I am having financial problems; and (6) Others.

Next, respondents without income and those who are in the low-income category (RM0-RM1000 per month) were asked to indicate the party paying for their follow-up costs and also the party they believe should be the payer. The follow-up costs here include consultation fees, blood tests, drug supplements, and miscellaneous. The payer and should be payer list was as follows: (1) Myself; (2) The kidney recipient; (3) My family members; (4) Charitable organization(s); (5) Private organization(s); (6) The government; and (7) Others.

Finally, we explored the respondents' views regarding their medical-institutional facilities. Respondents were asked whether they would like: (1) to be dealt with as patients, (2) to be under the supervision of 'donor clinic', (3) to be registered and monitored by living donor registry, or (4) to have an independent donor advocate. For each item, respondents were given three options 'Yes', 'No', and 'Undecided'. With respect to the organ donation-related institutions, the donors were told clearly about the donor clinic, donor registry and independent donor advocate.

This research was approved by the Medical Ethics Committee, University Malaya Medical Centre (**MEC Ref. No : 932.23**) on 19th July, 2012.

Results

Table 1 reports the respondent's social and economic background. Among the 80 donors, 57 (71.3%) were regularly attending their follow-up, 18 (22.5%) were non-regularly attending, and 5 (6.3%) had never attended the follow-up.

Table 1. Respondents' background (n=80)

Gender	Female	64%
	Male	36%
Marital status	Married	80%
	Single	16%
	Divorced	4%
Age	Below 40	18.75%.
	41-55	40%
	Above 56	41.25%
Income per month	No income	33.80%
	Below RM3000	51%
	RM3000 – RM4000	11.25%
	Above RM4000	3.75%

As shown in Table 2, about 30% of respondents consider themselves healthy and thus, consider themselves not in need of attending the follow-up. The second top cited reason for not attending the follow-up sessions was 'I don't want to take the trouble' (28.1%); while the other reasons captured lower attention. Under 'Others' option, all respondents explained that they could not come for the follow-up because they were busy with their work.

Chi-square tests showed no differences in the reasons given among the three categories of respondents (P>0.78). The reasons mentioned by the respondents are both hypothetical (for the regular and non-regular attendees) and actual (for those who never attended).

Table 2: Reasons for not attending follow-up sessions.

Reasons	Regular (n=57)	Non-regular (n=18)	Never (n=5)	Total (n=80)
1. I am just as healthy as anybody I know	33 (28.9%)	10 (27.8%)	4 (40.0%)	**47** (29.4%)
2. The doctor did not advise me to do so	17 (14.9%)	7 (19.4%)	2 (20.0%)	**26** (16.3%)
3. I do not want to take the trouble	33 (28.9%)	10 (27.8%)	2 (20.0%)	**45** (28.1%)
4. I do not want to be used as a research object	4 (3.5%)	1 (2.8%)	0 (0.0%)	**5** (3.1%)
5. I am having financial problems	14 (12.3%)	5 (13.9%)	0 (0.0%)	**19** (11.9%)
6. Others	13 (11.4%)	3 (8.3%)	2 (20.0%)	**18** (11.3%)
Total	114 (100.0%)	36 (100.0%)	10 (100.0%)	**160** (100.0%)

The results revealed that the majority of donors bore the costs of the follow-up sessions themselves (32.4%), while 25.0% were funded by their family members. The government funded the follow-up of only 17.6% of the donors, while the bulk (60.3%) of donors believe that the government should be the payer (Table 3).

The chi-square test revealed significant difference between the respondents' actual payer and 'should-be' payer, according to the donors' perception (P<0.01).

Table 3: Payer and 'should be' payer of follow-up costs for low and no-income group donors

Payer	Actual payer (n=68)	'Should-be' payer (n=68)	Chi-square test P-value
Myself	22 (32.4%)	10 (14.7%)	0.03
The kidney recipient	13 (19.1%)	4 (5.9%)	0.03
My family members	17 (25.0%)	10 (14.7%)	n.s*
Charitable organization(s)	1 (1.5%)	1 (1.5%)	n.s
Private organization(s)	1 (1.5%)	0 (0.0%)	n.s
The government	12 (17.6%)	41 (60.3%)	0.00
Others	2 (2.9%)	2 (2.9%)	n.s

*n.s: not significant

As illustrated in Table 4, more than two thirds of the respondents refused to be treated as patients. On the other hand, the bulk of respondents (more than 80%), showed an interest in having special donor registry and donor clinics to manage and monitor their health status after donation. However, only 40% of donors said 'yes' for having independent donor advocate.

Table 4: Donors' views on treatment, facilities and transplant institutions

Views	Yes	No	Undecided
I should be considered/treated as a patient too	15 (18.8%)	54 (67.5%)	11 (13.8%)
The government should set-up clinics for donors	64 (80.0%)	3 (3.8%)	13 (16.3%)
All donors should be systematically registered and monitored (living donors registry)	71 (88.8%)	3 (3.8%)	6 (7.5%)
I need to get advice from an independent individual who can represent and voice my interest (independent donor advocate)	32 (40.0%)	23 (28.8%)	25 (31.3%)

Discussion

The results showed that Malaysians who are living donors have a high frequency of follow-up attendance, in which about 71 % of respondents were regularly attending the follow-up. However, we cannot build a strong conclusion that the Malaysian living donors have higher follow-up commitment than their counterparts in other countries, given that only 80 donors out of 178 participated in this study.

Although the level of attendance among the respondents appears high, the majority of them seem to be unaware about the importance of the follow-up. This finding is shown by statistics, in which 29% of respondents stated that they might not need to pursue the follow-up sessions because they are as healthy as the new-donors. What confirmed the last mentioned idea is that another 29% of respondents considered the follow-up attendance as 'troublesome'. Another 11% believed that their work could be more important than the follow-up. Given these facts, we believe that there should be serious and urgent awareness campaigns targeting the living donors so as to educate them on the importance of the follow-up for their post-donation health status.

Although only 12% of respondents cited financial costs as the reason/could-be-reason of not attending the follow-up, the majority of the low and no-income respondents were funding their own follow-up costs. Nonetheless, the majority of donors felt that the financial burden of the follow-up was to be the duty of the government. The results showed that there is incongruity between expectation and reality. In the light of these results, omitting 2012 living donors from receiving free follow-up service might leave serious implications on the donor's life.

Therefore, we suggest that the government should bear the financial responsibility, and include all living donors as eligible to receive free follow-up service. The government should also take the initiative to establish special clinics for living donors and monitor donors' health under an official donor registry.

The donors should be duly informed about the existence and the roles of the independent donor advocate, so that the donors know about the source or entity to address if any possible grievances or problems arise in the future.

Conclusion

Even though majority studies have indicated that living donors are just as safe as other normal individuals both in morbidity and quality of life, the need to attend follow-up sessions should be emphasized by the government so as to avoid any unwarranted health issues. This also indirectly shows that the government takes care of the well-being of the donors.

Providing proper health care to the living organ donors is expected to increase the number of living donations. Prospective donors would more likely feel that their health status will be monitored and that they would not face any financial hardship after the donation. These efforts would have the potential to encourage all prospective donors to donate. Consequently, increasing the number of living donors could relieve a severe shortage of the pool of human organs in Malaysia.

A flexible system to accommodate the donors' availability in attending follow-up sessions should be introduced. Furthermore, a mandatory national living organ donors' registry ought to be established to keep track of the list of individuals who have donated organs. The role of the independent donor advocate should be structured holistically to ensure good service to the donors. Having the presence of the independent donor advocates alone is not good enough; their existence should be made known widely and they should be easily accessible.

Competing Interest

It is hereby declared that there is no conflict of interest pertaining to this paper.

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STEM CELL THERAPY AS A POTENTIAL TREATMENT FOR GLAUCOMA

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ABSTRACT

Glaucoma is a common eye disease that can cause irreversible damage if left undiagnosed and untreated. It is one of the most common neurodegenerative diseases causing blindness. Pre-clinical studies have been carried out on animal models of glaucoma for stem cell therapy. We carried out a systematic review to determine whether stem cell therapy had the potential to treat glaucoma. Nine studies were selected based on the predetermined inclusion and exclusion criteria. Of these nine studies, eight focused on neuroprotection conferred by stem cells, and the remaining one on neuroregeneration. Results from these studies showed that there was a potential in stem cell based therapy in treating glaucoma, especially regarding neuroprotection via neurotrophic factors. The studies revealed that a brain-derived neurotrophic factor expressed by stem cells promoted the survival of retinal ganglion cells in murine glaucoma models. The transplanted cells survived without any side effects. While these studies proved that stem cells provided neuroprotection in glaucoma, improvement of vision could not be determined. Clinical studies would be required to determine whether the protection of RGC correlated with improvement in visual function. Furthermore, these murine studies could not be translated into clinical therapy due to the heterogeneity of the experimental methods and the use of different cell lines. In conclusion, the use of stem cells in the clinical therapy of glaucoma will be an important step in the future as it will transform present-day treatment with the hope of restoring sight to patients with glaucoma.

Keywords: *Glaucoma, stem cell therapy, transplantation, neuroregeneration and neuroprotection, tissue degeneration, Regenerative Medicine.*

Introduction

Glaucoma is an eye disease that can cause irreversible damage if left undiagnosed and untreated (1). It is one of the most common neurodegenerative diseases causing blindness which affected 60.5 million people in 2010 and is predicted to increase worldwide to 79.6 million by 2020 (2).

Glaucoma is characterised by progressive ganglion cell and optic nerve (ON) damage leading to constriction of the visual fields and later to the loss of central vision. Although it is usually associated with increased intraocular pressure (IOP), there are patients with low or normal tension glaucoma. There are also others with increased IOP, who do not experience optic nerve damage. Increased age and elevated IOP are the risk factors for developing glaucoma. Normal IOP is 10 to 21 mmHg, but it can exceed 70 mmHg in glaucoma (3). Elevated IOP is due to increased accumulation of extracellular matrix material in the trabecular meshwork (TM) as a result of an imbalance between extracellular matrix deposition and degradation (4). Among the pathological changes that occur in glaucoma are neural tissue loss, microglia activation, tissue remodelling and disturbance of blood flow. These initial damages affect the ON axons (5). In the early stages of glaucoma, there is a progressive loss of the neural cells of the eye such as retinal ganglion cells (RGCs) and supporting cells, such as retinal pigment epithelium (RPE) (6). RGCs die in glaucoma, causing ON degeneration and disrupting the retinal connection to the brain. Neuronal death in

glaucoma starts with a primary axonal injury, followed by the death of injured neurones and ends with secondary degeneration of neighbouring intact neurones. The neural retina and ON are not capable of regeneration. Therefore, at present, loss of vision in glaucoma is irreversible.

Glaucoma in many patients progresses despite treatment (7). First-line treatment includes topical selective or nonselective beta-blockers that reduce aqueous humor secretion or a topical prostaglandin analog which increases aqueous humour outflow from the eye (8). Second-line drugs are alpha-agonists and topical carbonic anhydrase inhibitors which reduce the secretion of aqueous humour. Third-line treatment options are parasympathomimetic agents such as pilocarpine, which also increases the outflow of aqueous humour from the eye. However, it causes pupil constriction resulting in blurred vision if there is central lens opacity. These drugs are not successful in all cases of glaucoma as they only delay the loss of vision, and without treating the underlying cause (1).

For patients who do not respond to anti-glaucoma medications, laser therapy may be provided. With laser trabeculoplasty, the TM is treated with argon or diode laser, resulting in a rejuvenation of the TM. This treatment is mainly used in elderly patients because the effect is short term. Other procedures include iridotomy with an Nd: YAG laser, iridoplasty with an argon laser and ciliary body ablation where the laser burns the aqueous producing ciliary body and is used only in advanced refractory glaucoma (8). When there is a failure in medical or laser therapy to achieve the appropriate IOP, surgery may be offered as a treatment choice. Incisional surgery involves opening drainage channels in the anterior segment of the eye to lower the IOP (9). Trabeculectomy is the most efficient glaucoma filtration procedure. However, postoperative scarring of the drainage channel causes failure of the treatment. It can be prevented by using anti-scarring drugs such as 5-fluorouracil and mitomycin-c. The surgery also poses risks such as the development of postoperative cataract and the reduction in visual acuity (8).

Evidence obtained from studies on animal models suggests that stem cells are a potential new treatment for eye diseases, including glaucoma (4). The RGC, TM and the ON head are three potential targets for stem cell therapy in glaucoma. Most of the studies have focused on replacing RGCs because their death is the final common pathway for visual loss in glaucoma (7). Pre-clinical studies are being carried out in animal models of glaucoma. However, there is currently no literature review on whether these studies have been successful.

We performed a systematic review to evaluate the results of these studies and determine the feasibility if the studies can be applied to treat glaucoma in the future. Recent advances in stem cell based therapies hold the possibility of providing replacement cells for those cells damaged by glaucoma, as well as halting the disease progression via neuroprotection. If stem cell therapy can be used to treat glaucoma, it can reduce permanent damage and blindness in patients.

Methodology

This systematic review was a qualitative research based on an extensive literature search of publications in PubMed, Google Scholar and Cochrane Library by two researchers (FA and MK) who did the search independently. The keywords used for the literature search were generated using MeSH and included glaucoma, stem cells, progenitor cells, treatment and therapeutics. Animal studies using different cells to treat glaucoma either by neuroprotective or neurodegenerative methods were included in this study. Other inclusion criteria were English language publications, all study designs, and publications from 2000 to 2016. Studies on stem cell therapy for eye conditions other than glaucoma were excluded.

The extracted data from the selected studies were entered into a data extraction table. The data included names of authors, titles of journals, titles of studies, year of publication, study designs, sample sizes, type of cell used, interventions, comparison and outcomes. A comparative evaluation of the results of each study was also made.

Because only pre-clinical studies were available, a quality assessment could not be performed. Meta-analysis could not be done due to the heterogeneity of settings, interventions and outcomes. A narrative synthesis of the included studies was thus performed.

Study selection

A total of 4309 articles were retrieved, 1874 in PubMed and 2435 in Google Scholar using the keywords individually and in combination (Figure 1). A random hand search using a combination of words was then used. The abstracts of the articles with titles that could match most of the search criteria were read through. The article was included in the review once it met all the inclusion criteria. A total of nine articles were found suitable for analysis. One study addressed the neurodegenerative potential of stem cell therapy in glaucoma; seven studies looked at the neuroprotective effects of cell therapy in glaucoma and one study investigated both neuroregenerative and neuroprotective cell therapy. The details of the characteristics and the comparison of the outcome of the studies are presented in Table 1.

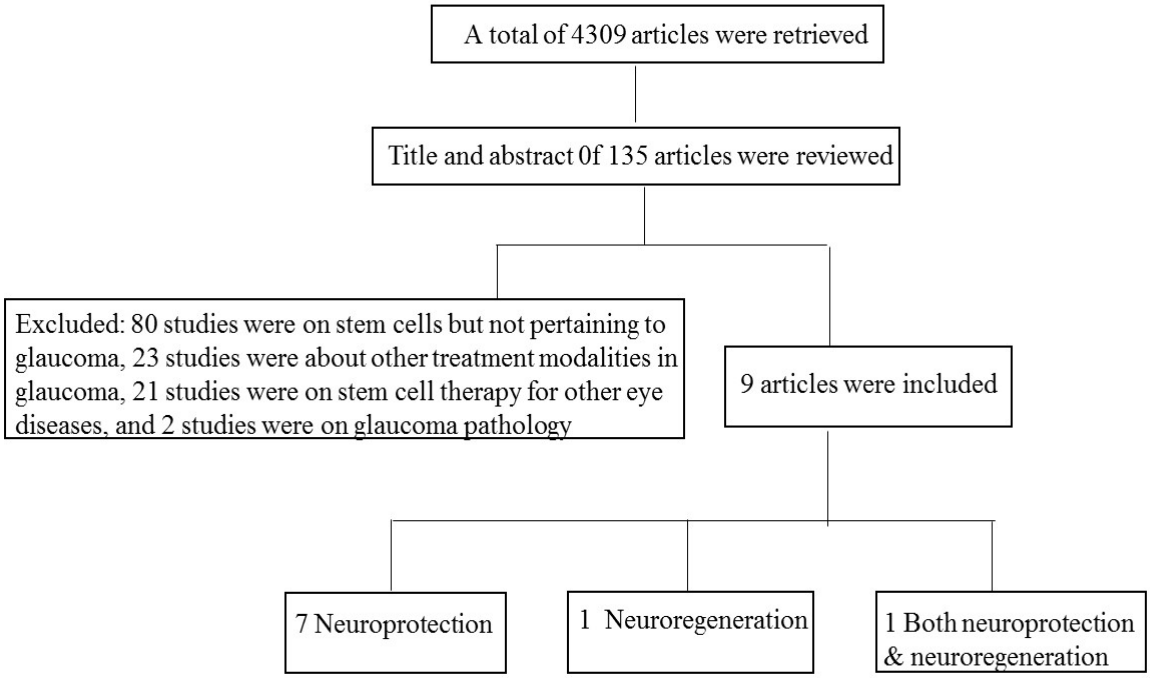

Figure 1: Flowchart of studies selection

Table 1: Selected preclinical trials of stem cells therapy in glaucoma model

No	Year	Author	Title	Study design	Population	Intervention	Comparison	Outcome
1	2008	Bull *et al*. (10)	Human Müller Stem Cell (MIO-M1) transplantation in a murine model of glaucoma: survival, differentiation and Integration.	Animal study	8 weeks old male Wistar rats	Intravitreal or subretinal stem cell injection with concurrent intraocular injection of 10 mU/eye chondroitinase ABC, *n*=17 or 200 ng/eye recombinant rat erythropoietin, *n* = 12.	Intravitreal stem cell injection, close to RGC layer, *n*=46. Subretinal stem cell injection, *n*= 42.	Intravitreally transplanted MIO-M1 cells were able to survive three weeks in vivo. Subretinally transplanted MIO-M1 cells could only survive for two weeks after injection due to invasion by immune cells. MIO-M1 cells that were transplanted either intravitreally or subretinally had difficulty migrating into the retina. MIO-M1 cells were able to infiltrate the retina with concurrent intraocular injection of either erythropoietin or chondroitinase ABC.

RGC retinal ganglion cell

Table 1: Selected preclinical trials of stem cells therapy in glaucoma model (continued)

No	Year	Author	Title	Study design	Population	Intervention	Comparison	Outcome
2.	2010	Johnson, *et al*. (11)	Neuroprotective Effects of Intravitreal Mesenchymal Stem Cell Transplantation in Experimental Glaucoma	Animal study	8- to 12-week-old male Sprague-Dawley (SD) rats and WT Lewis rats	Intravitreal MSC, n=20 (10 Lewis, 10 SD) Intravenous MSC, n=9 (9 SD)	Intravitreal dead MSC, n=19 (10 Lewis, 9 SD) Intravenous dead MSC, n=8 (SD) Intravenous PBS, n=8	Intravitreously transplanted mesenchymal stem cells (MSC) were found mainly in the vitreous cavity, with a small proportion of discrete cells migrating into the retina. Intravenous transplantation did not result in MSC migration to the retina. Lewis and SD rats that received intravitreal live MSC grafts exhibited higher RGC axon survival and lower rate of RGC axon loss compared with those that received dead MSCs. Intravenous injections of saline, live MSCs or dead MSCs, showed no significant difference in optic nerve damage.

MSC mesenchymal stem cells; SD Sprague-Dawley; RGC retinal ganglion cell

Table 1: Selected preclinical trials of stem cells therapy in glaucoma model (continued)

No	Year	Author	Title	Study design	Population	Intervention	Comparison	Outcome
3.	2006	Yu *et al*. (12)	Effects of Bone Marrow Stromal Cell Injection in an Experimental Glaucoma Model	Animal study	9 week old female Wistar rats	Intravitreal injection of GFP-BMSCs into the glaucomatous eye, n=69	Intravitreal injection of PBS into the glaucomatous eye, n=17	Intravitreal injection of BMSC into the glaucoma model eyes resulted in greater RGC densities compared to intravitreal injection of PBS. The majority of GFP-BMSCs within the retina were found along with the inner limiting membrane, while only a few cells integrated into the nerve fibre layer and ganglion cell layer. At both 2 and 4 weeks after transplantation, GFP-BMSCs that expressed CNTF, GDNF, BDNF, bFGF, and HGFα were observed in the glaucomatous retinas.

GFP-BMSCs Green fluorescent protein-bone marrow stromal cells; RGC retinal ganglion cell; PBS Phosphate-buffered solution; CNTF ciliary neurotrophic factor; GDNF glial cell line-derived neurotrophic factor; BDNF brain-derived neurotrophic factor; bFGF basic fibroblast growth factor; HGFα hepatocyte growth factorα

Table 1: Selected preclinical trials of stem cells therapy in glaucoma model (continued)

No	Year	Author	Title	Study design	Population	Intervention	Comparison	Outcome
4.	2009	Bull *et al*. (13)	Transplanted Oligodendrocyte Precursor Cells Reduce Neurodegeneration in a Model of Glaucoma	Animal study	8 weeks old male Lewis rats	Intravitreal transplantation at glaucoma induction: 3×10^4 live OPCs in PBS, n=10 Intravitreal injection before glaucoma onset: 3×10^4 live OPCs in PBS, n=9 or activated 3×10^4 OPCs in 3 µL PBS, n=9.	Intravitreal transplantation at glaucoma induction: 3 µL PBS, n=8, 3×10^4 dead OPC in PBS, n=10 Intravitreal injection before glaucoma onset: 3 µL PBS,n=10, 3×10^4 dead OPCs in PBS, n=10 Intravitreal injection of zymosan in 3 µL PBS, n=9	Transplanted OPCs were found to survive within the eye for 4 to 12 weeks. Activated OPCs significantly enhanced the survival of RGCs in the glaucomatous eye. Non-activated OPCs were not found to be neuroprotective. Transplanted OPCs differentiated into oligodendrocytes. They had a neuronal-like morphology and expressed bIII tubulin. They also differentiated into astrocytes as seen by astrocytic morphology and the presence of GFAP marker

OPC Oligodendrocyte Precursor Cells; PBS Phosphate-buffered solution ; GFAP Glial fibrillary acidic protein

Table 1: Selected preclinical trials of stem cells therapy in glaucoma model (continue)

No	Year	Author	Title	Study design	Population	Intervention	Comparison	Outcome
5.	2011	Harper *et al*. (14)	Transplantation of BDNF-Secreting Mesenchymal Stem Cells Provides Neuroprotection in Chronically Hypertensive Rat Eyes	Animal study	10 month old Brown Norway rats, n=28 E17 rat pups, n=36	Intravitreal transplantation of MSC engineered to express BDNF (BDNF-MSC)	Intravitreal transplantation of MSC engineered to express green fluorescent protein (GFP-) MSC	After transplantation, MSCs were mainly found adjacent to the GCL, integrated within inner retinal layers, or in the vitreous cavity. Eyes that received BDNF-MSCs displayed a greater level of RGC preservation than eyes that received GFP-MSCs. BDNF-MSCs preserved retinal electrical activity and had better afferent pupillary response compared to GFP-MSCs.

BDNF-MSCs brain-derived neurotrophic factor-mesenchymal stem cells; GFP Green fluorescent protein; RGC retinal ganglion cell; GCL ganglion cell layer

Table 1: Selected preclinical trials of stem cells therapy in glaucoma model (continued)

No	Year	Author	Title	Study design	Population	Intervention	Comparison	Outcome
6.	2012	Park *et al*. (15)	Stem Cell-Based Delivery of Brain-Derived Neurotrophic Factor Gene in the Rat Retina	Animal study	7-8 weeks old Sprague-Dawley rats	Single unilateral intravitreal injection of MSC with BDNF gene, n= 16 Single unilateral subretinal injection of MSC with BDNF gene, n=36.	Single unilateral intravitreal injection of PBS, n=16 or MSC, n=16 Single unilateral subretinal injection of PBS, n=31, or MSC, n=36	Subretinal injection of MSCs produced migration and integration into the rat retina. Some of the transplanted MSCs exhibited morphological changes, but differentiation could not be determined. Intravitreal injection of MSCs were clustered in the vitreous cavity, and integration into the retina was not detected. BDNF mRNA and protein levels were significantly increased in rats that received subretinal injection of transduced MSCs compared with those that received intravitreal injection of transduced MSCs, non-transduced MSCs and PBS.

MSCs mesenchymal stem cells; BDNF brain-derived neurotrophic factor

Table 1: Selected preclinical trials of stem cells therapy in glaucoma model (continued)

No	Year	Author	Title	Study design	Population	Intervention	Comparison	Outcome
7.	2002	Wang *et al*. (16)	Protection of Retinal Ganglion Cells Against Glaucomatous Neuropathy by Neurotrophin-Producing, Genetically Modified Neural Progenitor Cells in a Rat Model	Animal study	Adult male Sprague-Dawley rats	Intravitreal transplantation of genetically modified neural progenitors producing BDNF (BDNF-NPC) into the left eye, n=5 Subretinal transplantation of genetically modified neural progenitors producing BDNF (BDNF-NPC) into the left eye, n=5	Intravitreal sham injection, n=5 Intravitreal injection of NPC into the left eye, n=5 Subretinal sham injection, n=5 Subretinal injection of NPC into the left eye, n=5.	Transplanted cells migrated to a large retinal area. BDNF was expressed by BDNF-NPC transplanted via both intravitreal and subretinal route. RGC density in the groups treated with intravitreous or subretinal injection of BDNF- NPCs was higher than in the groups treated with intravitreous or subretinal sham injection or NPC injection. RGC apoptosis was also lower in groups treated with BDNF-NPCs than in other groups.

BDNF-NPC Brain-derived neural factor – neural progenitor cells; NPCs neural progenitor cells; RGC Retinal ganglion cells

Table 1: Selected preclinical trials of stem cells therapy in glaucoma model (continued)

No	Year	Author	Title	Study design	Population	Intervention	Comparison	Outcome
8.	2013	Zhou *et al*. (17)	Neuro-protection of Retinal Stem Cells Transplantation Combined with Copolymer-1 Immunization in a Rat Model of Glaucoma	Animal study	9 weeks old female Sprague-Dawley rats	Immunization with COP-1 followed by intravitreal transplantation of RSC (COP-1/RSCs)	Immunization with COP-1 and PBS (COP-1/PBS) Injection of PBS and transplantation of RSC (PBS/RSCs) Injection of PBS followed by another injection of PBS (PBS/PBS).	Transplanted cells integrated into the retina. The expression of BDNF and IGF-I in the RSCs/COP-1 group was significantly higher than in other groups. The number of the apoptotic RGCs in the RSCs/COP-1 group was notably lower than in other groups. The number of RGCs in the RSCs/COP-1 group was higher than in other groups.

RSC Retinal stem cells; PBS Phosphate-buffered saline; COP-1 Copolymer-1; BNDF Brain-derived neurotrophic factor; IGF-1 Insulin-like growth factor 1

Table 1: Selected preclinical trials of stem cells therapy in glaucoma model (continue)

No	Year	Author	Title	Study design	Population	Intervention	Comparison	Outcome
9.	2015	Emre *et al*. (18)	Neuroprotective effects of intravitreally transplanted adipose tissue and bone marrow-derived mesenchymal stem cells in an experimental ocular hypertension model.	Animal study	Adult female, Wistar albino rats	BM-MSCs/AT-MSCs group were intravitreally transplanted to the eyes 2 weeks after OHT	BM-MSCs/AT-MSCs group were intravitreally transplanted to the eyes 4 weeks after OHT	The retinal ganglion cell numbers per area were significantly improved in stem cell treated OHT groups compared with that in the non-treated OHT group. A limited number of stem cells had integrated into the ganglion cell layer and the inner nuclear layer.

BM-MSCs bone marrow derived mesenchymal stem cells; AT-MSCs adipose tissue mesenchymal stem cells; OHT - Ocular hypertension

Discussion

The majority of studies in this review focused on neuroprotection conferred by stem cells, rather than neuroregeneration. Only the study by Bull et al. and Emre et al focused on human Müller stem cells (MIO-M1) in neuroregeneration. These cells are derived from a population of Müller glia. The MIO-M1 cells were transplanted after glaucoma induction. These cells have been shown to proliferate in vitro and have the ability to differentiate into many types of retinal neuron.

Intravitreal transplantation of employed cells was done by all 9 selected studies, but only two studies have also used subretinal transplantation and one study also used the intravenous injection of cells to evaluate any potential

effect of the systematic application of stem cells for glaucoma. The intravitreous route was found to be more effective than the subretinal route in terms of duration of cell survival. However, cells transplanted via both routes had difficulty migrating into the retina. Erythropoeitin and chondroitinase ABC were needed to increase cell migration into the retina. Erythropoietin facilitates cell integration into the retina by upregulating matrix metalloproteinase 2 expression in the central nervous system. It may also be able to enhance the migration of Müller cells in the retina. Chondroitinase ABC encourages plasticity by modifying the central nervous system environment.

Johnson et al. (2010) studied whether MSC could provide neuroprotection to RGC in glaucoma. A possible contributory factor to glaucoma is the impaired RGC retrograde transport of neurotrophic factors (11). MSC was chosen because they had been shown to provide neuroprotection in degenerative central nervous system models (19). Following transplantation, they secreted neurotrophic factors and anti-inflammatory cytokines. Autologous transplantation was also possible with MSC. at study showed that intravitreal transplantation of MSC provided neuroprotection. Local transplantation had a better neuroprotective effect compared to than systemic transplantation. Intravitreally transplanted MSC survived well during the five-week study, despite no immunosuppression provided. Long-term graft survival is very promising, as it means a single treatment may be able to confer sustained neuroprotection. Intravenous transplantation of MSC proved to be unsuccessful, in contrast to other studies involving intravenous MSC transplantation in central nervous system pathologies (20, 21). The authors hypothesised that this might be because there is was less inflammation in glaucomatous neurodegeneration as compared to other chronic pathologies. Thus, RGC death might not produce a chemoattractive signal to draw stem cells transplanted intravenously into the retina. Two different rat breeds were used in this study because different breeds had been noted to have a dissimilar inflammatory response and protective autoimmunity (22). However, in this study, there was no difference in RGC neuroprotection by intravitreal MSC transplantation between inbred Lewis and outbred SD rats. In previous studies, intravitreal transplantation of stem cells did not affect ocular hypertension. Thus, the observed difference that glaucomatous SD rats which received live MSCs had lower IOP than glaucomatous SD rats that received dead MSCs could be due to model variability. This effect was not seen in glaucoma induced Lewis rats. Nevertheless, the transplantation of dead MSCs possibly influenced IOP elevation in SD rats, or intraocular transplantation of live MSCs reduced IOP in SD rats. Further investigation is needed to confirm whether this is indeed true.

The study by Yu et al. (2006) showed that transplantation of bone-marrow stromal cell (BMSC) did not cause inflammation or retinal structure destruction (12). However, unlike the studies by Bull et al., the transplanted cells did not express neuronal or glial markers. Thus there was no sign of differentiation. In this study, BMSC did not differentiate possibly because it was difficult to integrate into the retina. Transplanted cells faced difficulty in migrating from the vitreous cavity into the retina due to the inner limiting membrane (ILM), and without proper integration, differentiation could not happen. Thus, it is important to create a method in which transplanted cells can penetrate the ILM. Although integration occurred at a low level, RGC was protected, most probably due to neurotrophic factors and not to the replacement of cells. The trophic factor BDNF stimulates axon growth and CNTF is an RCG survival factor.

In the study by Bull et al. (2009) on oligodendrocyte precursor cells (OPC), IOP was noted to be lower in rats with glaucoma induced at eight weeks of age, as compared to rats with glaucoma induced at 16 weeks of age (13), showing that age played a role in clinical glaucoma. The OPCs had to be activated first in order to provide neuroprotection. Inflammatory cell activation of OPC is not yet fully understood but is important as it seems to trigger remyelination. Trophic factors supplied by OPCs played a part in preventing RGC death because OPCs did not migrate into neural tissue and were not directly in contact with inner retinal neurones. Compared to stem cells, progenitor cells have the capacity to differentiate but have no self-renewal capability. They are also lineage-specific precursor cells with more limited development potential. Thus, progenitor cells are more restricted than a stem cell in the type of cell it can become. In this study, OPCs were found to differentiate into astrocytes and oligodendrocytes. However, astrocytes at the optic nerve head have been seen in animal models of glaucoma progression (23). This makes transplanting OPCs, which may produce astrocytes a questionable therapy. However, in this study, the transplanted cells did not localise to the optic nerve head. Thus, the ill-effects of astrocytes can be avoided. Furthermore, astrocytes also produce trophic factors.

The study by Harper et al. (2011) showed that intravitreally transplanted MSC engineered to produce brain-derived neurotrophic factor (BDNF) were able to survive in glaucomatous eyes (14). They were mainly found adjacent to the GCL, integrated within inner retinal layers, or in the vitreous cavity. BDNF-MSCs were found to protect the retina and optic nerve function more significantly compared to GFP-MSC when tested for pupillary light reflex and ERG. Eyes that were transplanted with BDNF-MSC also showed higher RGC preservation. However, there was no difference in optic nerve integrity between BDNF-MSC–treated eyes and GFP-MSC–treated eyes.

The study by Park et al. (2012) on MSC transduced with BDNF cDNA showed that subretinal injection had more promising results compared with intravitreal transplantation (15). These results were unlike the earlier study by Bull et al. on Müller stem cells, with more efficacious results with intravitreal transplantation, or the study by Wang et al. in which both intravitreal and subretinal transplantation

resulted in NPC migration to the retina. However, retinal folding and retinal splitting were seen following subretinal transplantation, though there was no damage at the injection site. Even though there were morphological changes in some of the transplanted MSCs, differentiation could not be determined. This could be because the yields of MSCs that differentiated into other types of retinal cells were not enough to provide a definitive result. The method used to induce a glaucomatous condition was also different from other studies, in that optic nerve axotomy was performed instead of laser treatment to the trabecular meshwork. Axotomy mimics the condition in glaucoma because the axonal transport of neurotrophic factors is blocked, as what occurs in increased IOP. The neurones that are deprived of these factors will then die. Whether or not the different methodology influenced the results would require more comparative studies.

Wang et al. (2002) also employed a condition which mimicked glaucoma, by partially blocking ON axoplasmic flow (16). This study found that transplanted NPCs were able to migrate from the point of transplant to a large area of the retina. The genetically modified NPC producing BDNF transplanted either intravitreally or subretinally were compared with sham injection and NPC injection. BDNF-NPCs were proven to be neuroprotective as there was a higher RGC density and less RGC apoptosis. There was also no sign of rejection.

Zhou et al. (2013) used RSC transplantation and vaccination with a glatiramer acetate copolymer-1 (COP-1) to study its effects on neuroprotection in a rat glaucoma model (17). In this study, many RSC were found to migrate to the retina and integrate into the nerve fibre layer and GCL. This result is better than that of the study by Harper et al., in which the transplanted MSCs were found adjacent to GCL and the study by Johnson et al., where most of the transplanted MSCs were in the vitreous cavity (11, 14). This was possibly due to the different types of stem cell used as well as the microenvironment suitable for cell migration. The number of the apoptotic RGCs in the RSCs/COP-1 group was also lower than in other groups, and the number of RGCs in the RSCs/COP-1 group was higher than in other groups. COP-1 immunization boosts the body's protective autoimmune response (17). The increase in levels of secreted BDNF and IGF-I could be one of the mechanisms conferring neuroprotection to RGCs.

A study by Emre et al. (2015) showed that MSCs from adipose tissue and bone marrow displayed similar neuroprotective effects and that the cells injected into the vitreous cavity were integrated into the retina (18). However, the study by Yu et al. reported that bone marrow MSC did not differentiate, possibly because it was difficult to integrate into the retina (12). Transplanted cells faced difficulty in migrating from the vitreous cavity into the retina due to the inner limiting membrane (ILM). In other words, without proper integration, differentiation could not happen. Thus, it is important to create a method in which transplanted cells can penetrate the ILM. Because integration occurred at a low level, but RGC was protected, the protection was most probably due to neurotrophic factors and not to the replacement of cells. The trophic factor BDGF secreted from MSCs, stimulated axon growth and RCG survival. In all these studies, the survival rate of injected cells was varied between 2 to 12 weeks.

One of the limitations of the selected studies in their methodology was due to the method of induction of glaucoma. Glaucoma was induced by increasing ocular hypertension (IOP) in eight studies. Wang et al. used a power forcep to crush the ON close to its origin in the optic disc to induce glaucoma in the rat. It is important to bear in mind that not all types of glaucoma are due to IOP and future studies may induce glaucoma in the animal model via other mechanisms which are known to cause glaucoma. Another limitation of this review is that clinical trials were not captured; as our aim was to describe the stem cell therapy based on the rat model alone.

Conclusion

There is potential in stem cell based therapy in treating glaucoma, especially in terms of neuroprotection via secretion of neurotrophic factors. The majority of the studies showed that BDNF expressed by stem cells was able to promote the survival of RGC in rat glaucoma models.

The transplanted cells such as MSCs can survive without any side effects. This is indeed beneficial since autologous transplantation of MSC can reduce the risk of rejection as well as avoid transmission of infectious agents from donors to recipients. Ethical concerns can also be avoided by using stem cell lines derived from adults, and not embryonic or fetal tissue. Compared to therapeutic agents which require repeated administrations, stem cell transplantation provides an extended expression of protective neurotrophic factors.

Many issues need to be addressed to overcome the problems of stem cell therapy in glaucoma. The molecular pathology of glaucoma and the consequences of increased IOP at the cellular level must first be fully understood. Assessment of safety is important to ensure that the transplanted cells do not impair visual function, as the cells tended to stay within the vitreous cavity. As stem cells retain their proliferative ability, further research is needed to ensure that tumours will not be generated by the grafted cells.

The findings of these studies cannot yet be translated into clinical therapy due to the mixed results obtained from different experimental models. As the field of stem cell research and application advances and our knowledge on stem cells increases, stem cells may be used not only to rescue but also to replace retinal neurones. The use of stem cells in the clinical therapy of glaucoma will be an important step in the future as it will transform present-day treatment with the promise of restoring sight to patients with glaucoma.

Future recommendations

Animal studies prove that stem cells can provide neuroprotection in glaucoma, but the clinical improvement of vision could not be assessed. This requires further clinical studies to determine whether the protection of RGC correlates with improvement in visual function. These limitations do not detract from the significance of the research findings, and should provide a platform for future research.

It is important that clinical trials be conducted on stem cell therapy in glaucoma. Even though the present studies utilised different types of stem cells, including retinal stem cells and neural progenitor cells, harvesting these stem cells may prove to be problematic clinically. Thus, future studies should focus more on MSC transplantation and expression of BDNF. The method of transplantation must also be refined, as pre-clinical studies have shown a discrepancy in the efficacy of intravitreal and subretinal transplantation.

Future studies should also look towards stem cells in adjuvant therapy, whereby stem cell therapy is combined with pharmacology, bio-engineering, or gene therapy. This may serve to increase the therapeutic functions of stem cells.

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PARKINSONISM AND BRAIN MRI FINDINGS IN A RELAPSED CULTURE-PROVEN *SALMONELLA TYPHI* INFECTION: A CASE REPORT IN MALAYSIA

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ABSTRACT

Typhoid fever is a systemic infection caused by *Salmonella typhi*, which may be associated with extra-intestinal complications. Neurological manifestations, particularly Parkinsonism, are rarely reported. We report a 17-year-old patient with relapsed culture-proven *Salmonella typhi* infection who developed septic shock and subsequently Parkinsonism. Lumbar puncture revealed acellular cerebrospinal fluid with raised protein level. Magnetic resonance imaging revealed cerebral petechial haemorrhages resulted from small vessels vasculitis. His symptoms resolved spontaneously after 3 months.

Keywords*: Typhoid fever, Salmonella typhi, neurological manifestation, Parkinsonism, MRI brain*

Background

Typhoid fever is a systemic infection caused by *Salmonella typhi* or *paratyphi A*. Headache is a common symptom in 44-94% of cases (1-3) but other neurological manifestations such as encephalopathy, meningitis, Parkinsonism, motor neuron disorders, ataxia, cerebral abscesses, cerebral oedema, seizures and Guillain–Barré syndrome are infrequently reported. This is a case report describing the clinical presentation and features of the magnetic resonance imaging (MRI) of the brain of a patient with relapsed culture-proven *Salmonella typhi* with Parkinsonism.

Case Report

A 17-year-old male presented to the Emergency Department with a 5-day history of high-grade fever as well as diarrhoea that lasted for 2 days. He was previously treated for typhoid fever and had completed a course of treatment with ceftriaxone one month prior to admission. On examination, he was febrile and had hepatosplenomegaly. A diagnosis of relapsed typhoid fever was considered and he was started on empirical intravenous (IV) ceftriaxone therapy.

Laboratory tests showed the following results: white blood cell count 4400/µl (neutrophils 62.4%, lymphocytes 29.9%, monocytes 7.2%); haemoglobin 13.6 g/dl; C-reactive protein 125.8 mg/dl; platelet count 37,000/mm^3; total bilirubin 41.8 µmol/l; aspartate aminotransferase 251 IU/l; alanine transaminase 109 IU/l; lactate dehydrogenase 1481 IU/l; creatine kinase 2418 IU/l; and normal renal function. Serum rapid HIV test was negative.

Blood culture taken at admission grew *Salmonella typhi* on day 2 of admission, with susceptibility to Ampicilin, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Trimethoprim/Sulfamethoxazole, Imipenem and Meropenem. He was continued on Ceftriaxone. His platelet count increased to a normal level since day 5 of admission following a total of 12 units of random platelet transfusion from day 2 to day 4 of admission. He had septic shock complicated by myocarditis and was transferred to the Intensive Care Unit. The patient received an initial bolus of high dose intravenous dexamethasone 3 mg/kg, followed by eight consecutive doses of the drug at 1 mg/kg administered 6-hourly. Subsequently, he developed nosocomial infection. On day 22 of admission, his fever settled but he was found to have bilateral pill-rolling tremor and cogwheel rigidity. Lumbar puncture was done and the result showed that his cerebrospinal fluid was acellular and had protein level of 876 mg/dl and glucose level of 3.9 mmol/dl (blood glucose 4.9 mmol/dl). The cerebrospinal fluid gram stain and culture were negative for bacterial and fungal growth. Magnetic resonance imaging (MRI) of

the brain performed on day 30 of hospitalization revealed multiple petechial haemorrhages in the brain parenchyma involving the junction between the grey and white matter, corpus callosum and internal capsule bilaterally [Figure 1]. His symptoms resolved spontaneously over a period of 3 months.

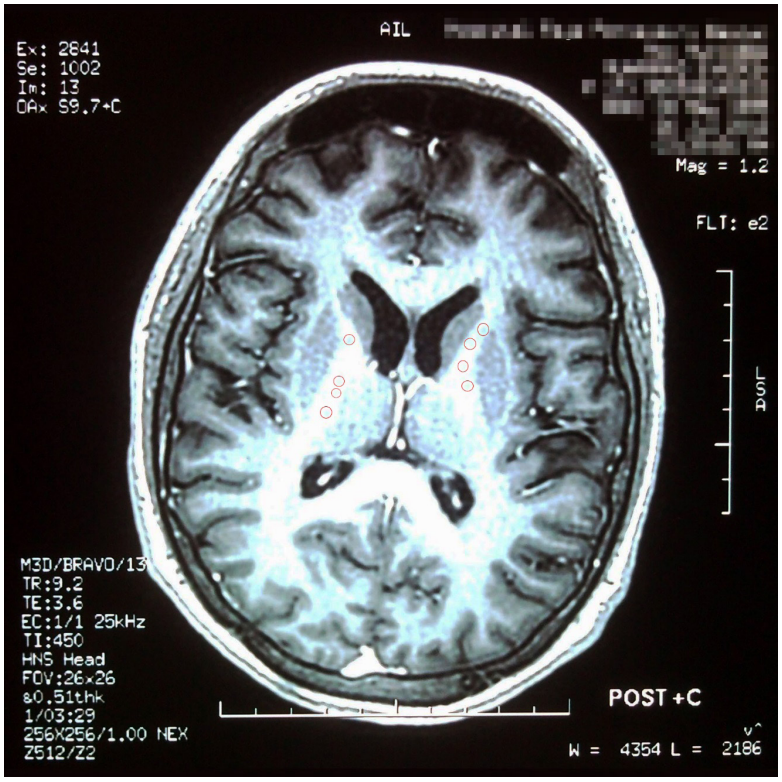

Figure 1: Axial T1W magnetic resonance image of the brain showing hypointense foci (red circles) in the internal capsule bilaterally. This finding is consistent with petechial haemorrhages.

Discussion

Typhoid fever is caused by facultative gram-negative bacillus *Salmonella typhi* or *paratyphi A*. It usually occurs in developing countries as a result of poor sanitation and poverty. The prevalence of the disease is estimated to be 12-33 million cases (4). It can affect multiple organs system, and therefore, neurological sequelae is not uncommon. Relapse typhoid fever occurs in 5-10% of cases and is usually less severe than the initial episode. However, hepatomegaly is a more common sign in relapse cases (5).

Parkinsonism is a rare neurological manifestation of typhoid fever, which was first described by Millis in 1927 (6). In a study by Ali et al., 84% of cases of multidrug-resistant typhoid fever developed neuropsychiatric manifestations, which include acute confusional state (73%), myelitis (6%), cerebellitis (1%), Parkinsonism (1%), acute psychosis (0.6%), meningoencephalitis (0.5%), encephalitis (0.25%) and others [3]. During an outbreak in Malawi-Mozambique in the year 2009, 40 out of the 303 identified cases were associated with neurological abnormalities but only 8 of them had Parkinsonism (7). Neurological symptoms usually appear during the first few days of the fever but may occur up to the third week or during the convalescence period, as seen in this case.

The exact pathophysiology of central nervous system involvement in Salmonella typhi infection is not clear, and various mechanisms such as neuro-endotoxin interaction and altered immune response have been proposed (3). Everest et al. suggested that both bacteria and host response are crucial in the mechanism of severe typhoid infection. Immune response triggered by bacteraemia may lead to necrosis of the venules and capillaries, and finally, haemorrhage (8). Subthalamic dysfunction is associated with Parkinson's disease. Subthalamic activity is regulated by direct (through pallidothalamic) and indirect (through pallidosubthalamic) signal pathways out of the striatum. Pallidothalamic fibers pass through the internal capsule and lesions in this region may affect the balance between these two pathways, leading to Parkinsonism (9). This may explain the MRI findings of small vessel petechial haemorrhage involving the internal capsules in our patient.

Previous studies have reported various features from MRI investigation, ranging from normal appearance, demyelinating changes, symmetrical diffuse abnormal

signal with central area of restricted diffusion to diffuse cerebral oedema (10-13). The literature on imaging findings of Parkinsonism is lacking. Talukdar et al. reported a normal appearance on MRI in a patient with catatonia and Parkinsonism (13), however, the MRI was done prior to the onset of Parkinsonism. To the best of our knowledge, this is the first case report demonstrating the MRI findings in a patient with typhoid fever and Parkinsonism.

In the present case, the patient's neurological symptoms resolved spontaneously over a period of 3 months. A recent case report indicated that a combination of amantadine and dopamine agonist resulted in a complete recovery of symptoms within a month of treatment (13). High-dose intravenous methylprednisolone has also been shown to be successful in treating patients with salmonella-associated encephalopathy (11); however, its benefit in Parkinsonism is yet to be evaluated.

Conclusion

Parkinsonism is a rare neurological manifestation, which may be a complication of relapsed typhoid fever. Petechial haemorrhages of the brain parenchyma affecting the subthalamic activity may be one of the mechanisms involved. Parkinsonism may resolve spontaneously but antiparkinsonian therapy could facilitate recovery from this condition.

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Competing interests

All authors declare that they have no competing interests.

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