ASSESMENT ON THE CYTOTOXICITY, APOPTOSIS AND AUTOPHAGY PROPERTIES OF CHEMICALLY SYNTHESISED SILVER NANOPARTICLES ON BEAS 2B CELLS

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LIST OF SYMBOLS AND ABBREVIATIONS

CO_2	Carbon dioxide
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide
PBS	Phosphate buffered saline
FBS	Fetal bovine serum
DMSO	Dimethyl sulfoxide
PI	Propidium iodide
EDTA	Ethylenediaminetetraacetic acid
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'tetraethylbenzimidazolylcarbocyanine iodide
HIV	Human immunodeficiency virus
h	Hour
µl	Microlitre
µm	Micrometer
nm	Nanometer
ml	Millilitre
°C	Degree celcius
mg	Milligram
µg	Microgram
µM	Micromolar

mM	Millimolar
Bak	Bcl-2 Homologous Antagonist/Killer
Bax	Bcl-2-associated X protein
Bcl-xL	B-cell lymphoma extra large
Akt	Protein kinase B
mTOR	Mammalian target of rapamycin
Rictor	Rapamycin-insensitive companion of mTOR
PI3K	Phosphatidylinositide-3-kinase
g	Gram
%	Percent
DR	Death receptor
nM	Nanomolar
GSH	Glutathione
ROS	Reactive oxygen species
γ	Gamma
Δψm	Mitochondrial membrane potential

ABSTRACT

Silver nanoparticles (AgNPs) has been shown to exhibit anti- microbial effect. The exploitation of AgNPs has emerged in the recent years and may trigger major health problem. The effect of AgNPs on the human normal lung cells have not been studied. Here, we report on the chemically-synthesised AgNPs-induced cytotoxicity in human normal lung cells. In this study, chemically-synthesised AgNPs were evaluated on their cytotoxicity effects in Beas 2b human normal lung cells by measuring the cell viability, mitochondrial membrane potential, autophagy and apoptosis. The cell viability of AgNPs-treated cells was found to decrease in time- and dose-dependent manner. AgNPs also found to reduce the mitochondrial membrane potential of Beas 2b cells, which is an early indicator for the activation of apoptosis and mitophagy. Co-treatment of AgNPs with BEZ-235, a PI3K/mTOR inhibitor, has also increased the percentage of autophagy activity in Beas 2b cells. Prolong exposure of Beas 2b cells to AgNPs has also significantly increased the number of total apoptotic cell death. Therefore, this study suggest that exposure of chemically-synthesised AgNPs, particularly at higher doses with prolong incubation period, has induced cytotoxic effects in Beas 2b human normal lung cells.

ABSTRAK

Nanopartikel perak (AgNPs) telah dinyatakan mempunyai kesan anti mikrob. Eksploitasi AgNPs telah meningkat sejak tahun-tahun kebelakangan ini dan mungkin akan menimbulkan masalah kesihatan besar. Kesan AgNPs pada sel-sel paru-paru normal manusia masih belum diketahui. Dalam kajian ini, kesan-kesan sitotoksik terhadap sel manusia paru-paru normal Beas 2b diaruh oleh AgNPs yang disintesis secara kimia ditentukan dengan mengukur kadar pertumbuhan sel, potensi membran mitokondria, autofagi dan apoptosis. Kadar pertumbuhan sel yang dirawat oleh AgNPs didapati berkurang dengan pertambahan masa inkubasi dan kepekatan AgNPs. AgNPs juga didapati mengurangkan potensi membran mitokondria yang merupakan penunjuk awal bagi pengaktifan apoptosis dan mitofagi. Rawatan bersama AgNPs dengan BEZ-235, yang merupakan inhibitor bagi laluan PI3K / mTOR, juga meningkatkan peratusan aktiviti autofagi dalam sel Beas 2b. Penambahan masa rawatan dengan AgNPs juga meningkatkan jumlah kematian sel secara apoptosis dalam sel Beas 2b. Oleh itu, kajian ini menunjukkan bahawa pendedahan AgNPs yang disintesis secara kimia, terutamanya pada dos yang lebih tinggi dan tempoh inkubasi yang panjang, boleh mengaruh kesan sitotoksik terhadap sel manusia paru-paru normal Beas 2b.

CHAPTER 1

1.0 INTRODUCTION

Nanotechnology and nanoparticles are dynamically recognized for their conceivable use in advanced plane design, nanoelectronics, natural remediation, therapeutic care and customers merchandise. Nanoparticles are particles with minimun of one dimension ranging between 1 to 100 nm. Generally, the small size of the nanoparticles provides a greater expanse for the particles hence increasing the effect (Prabhu et al, 2012). It is reckoned that of all of the consumer goods with nanoparticles, silver nanoparticles (AgNPs) utilisation is at present having the broadest scale of commercialisation (Ahamed et al,2010).

AgNPs are used in finishing or mending for medical intention besides being utilised in the clothing wear, the food industry, paints, electronic parts and other areas (Piao et al,2010). To convey the antimicrobial effect, it is usually utilised in the nitrate form as there is an increase in the surface area approachable for the microbes to be open to compared to silver. AgNPs is able to grab hold and penetrate the bacterial cell wall thereby inducing changes in the cell membrane and eventually leading cell death. The use of AgNPs are also applied in bone filling which are utilised as an bionic joint replacement. AgNPs are also added to the fabricating inserts to reduce the wear and tear of the polymer. AgNPs can be thought-through as a possible choice for surgical

mesh as mesh coated with AgNPs is known to have a good anti-microbial activity (Prabhu et al, 2012).

However, the increase in the use of nanoparticles in consumers products has raised concerns that nanoparticles may be a risk to humans and the environment. The increase in the surface properties is due to to the high surface to volume ratio of nanoparticles therefore increasing the reaction formed. AgNPs with anti-microbial action can block the biological process of various ‘friendly’ bacteria in the environment which in turn can cause rivers and lakes eutrophication and damage the ecosystem (Hussain et al, 2005). High amount of silver which is released into the environment can in turn affect humans. The adverse effect of silver on living being includes permanent discolouration of the skin and the toxic effects to liver and kidney, intestinal tract and changes in blood cells (Hussain et al, 2005). It was reported aggregation of AgNPs in the liver could encourage cytotoxicity via oxidative cell damage (Hussain et al, 2005, Piao et al,2010). AgNPs also proved to be toxicant in-vitro mouse germ line stem cells as it diminished the mitochondrial function and caused leak through the cell membrane (Prabhu et al, 2012). Based on Piao et al (2010), studies done on human liver cells shows that AgNPs have shown to induce reactive oxygen species (ROS) generation and the depletion of intracellular GSH leading to the damage of the cellular components which in turn leads to cell death through the mitochondria-dependent and caspase dependent pathways.

Apoptosis which is a programmed cell death is a part of normal physiological means and is regulated in an organized fashion through a series of cascade which leads to the cell death. Apoptosis has a role in tissue homeostasis for proper development in regulating growth as well as a role in the defence mechanism in immune response where it destroys cells that represent a risk to the integrity of the organism (Okuichi *et al*., 2007). Apoptosis are characterized by the biochemical events which lead to the changes in morphological features such as the condensation of chromatin, shrinkage of cell, membrane blebbing, fragmentation of DNA and the apoptotic bodies formation (Okuichi *et al*., 2007). Generally, apoptosis starts with the damage of cells due to stress or other signals inside and outside of the body where cells then began to shrivel and form blebs. Cellular proteins are then activated to break down the cellular components where the nucleus will be broken down by enzymes and emit signals to engage the macrophages. The cells break into smaller pieces consisting of the cell components and destroyed nucleus attaractinf macrophage to recognize the cell surroundings and removes it from the body (Friedlander, 2003).

Although there are a few research literatures that propose that the nanoparticles can lead to damage to the environment and health, little is known about the mechanism of nano-silver. Therefore, there is still a need for studies to be conducted *in vitro* to determine its nanotoxicity effect on human

particularly at the cellular level. This study is conducted to investigate the cell death effects induced by chemically-synthesised AgNPs towards Beas 2b normal human lung cells. The findings obtained in this study may clarify and benefit towards the understanding of AgNP-induced effects related to human health risks.

CHAPTER 2

LITERATURE REVIEW

2.1 Nanoparticles

Nanoparticles (NPs) are defined as particles which are small in size with dimensions ranging between 1 to 100 nm. Nanoparticles possess a special chemical and physical characteristics compared to the main majority substance,which leads to progressive magnetic, electrical, optical, mechanical and composition properties (Beer et al., 2011). Nanomaterials also have been extensively used in the current years in many different fields for example medical healthcare and consumer products (Xin et al., 2015).

However, due to advantages also that the nanoparticles (NPs) are currently being exploited in the new product which have lead to concerns that NPs may endanger humans and environment. High surface area to volume ratio increases surface properties of the nanoparticle making it more interactive than larger particles besides enhancing the metal ion to be released from the nanoparticles (Bian et al, 2011, Mudunkotuwa et al, 2011;). All these have raised concerns that NPs may also interact in newfound ways with the natural systems (Beer et al, 2011, Landsiedel et al., 2010; Maynard et al., 2011; Oberdörster et al., 2005). In studies by Castranova et al., 2011 and Schrand et al., 2010, the toxicological investigation of NPs shows that the size, shape, chemical constitution, surface charge, solubility, its ability to bind and affect biological sites as well as their metabolism and elimination plays a role in the

toxicity of NPs.

2.2 Silver Nanoparticles (AgNPs)

Out of all the nanomaterials available, silver nanoparticles (AgNPs) have gained popularity due to its wide range of uses in antimirobial activity (Xin et al, 2015). It is widely used in medical products like bandages, coating of the surgical knives, as well as in textiles and household items such as food storage bags and refridgerator surfaces (Chaloupka et al., 2010, Marambio-Jones and Hoek, 2010).

Due to their distinctive bacterial properties, new applications in biomedicine and healthcare have been brought about. AgNPs can induce rapid healing where its antimicrobial properties improves cosmetics appearances, modulate cytokine expression and reduce inflammation (Tian et al, 2007). AgNPs have also been used as a coating on sugical knives to maintain sterility and reduce contamination and infection (Ong et al, 2013).

AgNPs are used in photoacoustic imaging and image guided therapy besides being used as a Surface Enhanced Raman Scattering (SERS) nanoprobes because of their solid optical absorbance and dispersing properties (Homan et al, 2010, Kneipp et al, 2010). A study was reported where the use of AgNPs and a chemotherapeutic agent caused a a noteworthy decline in the cell feasibility contrasted with the treatment of AgNPs and the chemotherapeutic agent separately (Mahmood et al, 2010). Eventhough there is no evidence that shows the significant differential response of the normal and cancer cell to the treatment, there is possibility that AgNPs can be used a

drug delivery agent.

Studies have shown that AgNPs is able inhibit VEGF cell multiplication, movement and narrow like tube development in bovine retinal endothelial cells (BREC) (Gurunathan et al, 2009, Ong et al, 2013). AgNPs also inhibited growth of mouse fibrosarcoma cell in a dose and time dependent manner suggesting the prospect of AgNPs to curb the development and proliferation of tumour cells in cancer therapy (Nallathamby, 2010).

2.2.1 Toxicity of Silver Nanoparticles (AgNPs)

AgNPs are toxic to many tissues including the brain, liver, lungs and vascular systems as well as reproductive organs. Although AgNPs is known for its advantages mainly in the use as a anti-microbial compound, there are also reports that states the toxicity in various species and high exposure to silver can cause argyria and/or argyrosis in humans (Bilberg et al., 2011, Drake and Hazelwood, 2005, Navarro et al., 2008). AgNps can be exposed through various ways such as inhalation dermal contact and oral ingestion where absorbed AgNPs is able to pass through the respiratory or the gastrointestinal tracts and distributed in various organs such as the lungs, liver and brain (Gaiser et al, 2013, Hagens etal, 2007). However, very little is know about the rate of dissolution of AgNPs (Liu et al, 2010). It is highly important for any microbial applications of AgNPs to determine the rate as it directly determine the concentration of the silver available in the AgNPs surrounding (Kittler et al, 2010).

Many studies was done to study the effect of AgNPs on inflammatory response. It was reported that AgNPs were able to initiate immune response in human monocytic cell line THP-1 (Martínez-Gutierrez et al., 2012). Park et al. (2010) reported that inflammatory responses were significantly promoted by repeated oral administration of AgNPs which was indicated by the increase in cytokine production, B- cell distribution and inflammatory cell filtration (Park et al., 2010). However, most reports were carried out in-vitro. Therefore more

evaluation should be performed to study the effect of AgNps on the immune system especially at the level of *in vivo*.

Studies have also address the toxicity effect of AgNPs in vitro (Foldbjerg et al., 2011, 2009; Kawata et al., 2009; Kim et al., 2009). However, it is still not clear how much the silver ions released will result in the toxicity of AgNPs. It was reported that oxidative stress by lipid peroxidation, protein oxidation and DNA damage were the major cause of AgNPs toxicity and the free silver ions as well as the calculated silver ions amount in the AgNP suspension is unable to justify the monitored effect of the toxicity of AgNPs in full (Kawata et al, 2009, Kim et al, 2009). Besides that, AgNPs also has shown potential in the handling of diseases that need maintenance for transporting drug concentration or for targeting specific cells or organs (Ahamed et al, 2010, Moghimi et al, 2001, Panyam et al, 2003). It was shown, for instance, that AgNps interacts with the HIV-1 virus and inhibit its ability to bind to host cells in vitro (Elechiguerra et al. 2005).

In vitro work have shown the potentiality of AgNps to cause tocixity in cells from various organs. For example the potential increase of skin exposure due to the use of AgNPs in cosmetics and textiles. A study was reported where using artificial human skin, silver can be released from anti-bacterial fabric product into sweat (Kulthong et al. 2010). The release of silver is depending on the pH, sweat formulation, fabric quality and the quantity of silver coating (Ahamed et al, 2010). The exposure of keratinocytes with skin wound dressing

containing silver extracts shows that it was among the cytotoxic (Paddle-Ledinek et al, 2006). Besides that, AgNps crystals from dressing were found to be toxic in both keratinocytes and fibroblats (Poon et al, 2004). It was also reported that AgNPs can cause cell death and oxidative stress in human fibrosarcoma and skin carcinoma cells besides being able to enter the cells and cause DNA damage and apoptosis in fibroblasts and liver cells (Arora et al, 2008, 2009). In a study by Hsin et al, the possible mechanism of AgNPs toxicity in fibroblasts was elucidated where it was found that reactive oxygen species (ROS) were induced and cytochrome c released into the cytosol and translocation of Bax protein into the mitochondria indicating the mitochondria-dependent apoptosis pathway in fibroblast (Hsin et al, 2008, Gopinath et al, 2010).

There are also evidences which display that AgNps are now able to have impact human reproductive system via various commercialized products. In a study by Braydich-stolle et al, AgNPs, via the reduction in the the function of mitochondria and membrane leakage induction and apoptosis, caused harmfulness to germ line stem cells (Braydich-stolle et al, 2005). Ahamed et al. (2008), also examined the DNA change reaction response to polysaccharide surface coverd and uncoated AgNPs in two murine mammalian cells, embryonic stem cells and embryonic fibroblasts and found that both assortment stimulated apoptosis through up-regulation of cell cycle protein p53 .

2.2.1.1 Toxicity of Silver Nanoparticles (AgNPs) on Lungs

Studies have demonstrated that liver and lungs are the real target tissues for delayed AgNPs exposure (Takaneka et al, 2001, Sung et al, 2008, Ahamed et al, 2010). In a study by Takaneka et al (2001), the investigation of the pulmonary and the systemic dissemination of silver particles in rates demonstrates that the substance of the lung particles decreased quickly with time upon inhalation and the particles were likewise discovered in other organs including the liver, kidney and brain. Tang et al (2009) found that when it was subcutaneously injected into the rates during the toxicity study, AgNPs was translocated to the circulation distribute to other organs.

Inhalation toxicity study by Jing et al. (2007) showed no noteworthy changes in the hematologic and biochemical esteems in short term. However, the tidal and minute volume and the inflammatory response of the lung was reduced in a long term study. It was reported that a significant depletion of antioxidant gluthathione, reduced mitochondrial membrane potential and increased ROS was observed in study of AgNPs on rat liver cells suggesting the cytotoxicity is most likely mediate through the oxidative stress in liver cells (Hussain et al, 2005). Non cytotoxic doses (0.5 μg/ml) of AgNps caused toxicity in the human mesenchymal stem cells and induced the gene expression links with the cell cycle progression and apoptosis in human hepatoma cells (HepG2) indicating that AgNPs are not safe even at non-cytotoxic doses (Kawata et al, 2009).

Studies by inhalation exposure suggested that lungs are the vulnerable target for nanoparticles and that through the nasopharyngeal system, it may reach the brain (Oberdorster et al, 2004). Studies have reported that inhale nanoparticles are likely to be deposited in the nasopharyngeal region and be translocated into the brain (Oberdorster et al, 2004, Elder et al, 2006). Hussain et al. reported that AgNps caused the dopamine depletion and cytotoxicity in neuroendocrine cells (Hussain et al, 2006). In a study by Soto et al, the cell viability of alveolar macrophages and lung epithelial cells were reduced by Ag NP and the oxidative stress mediated size-dependent toxicity of Ag NP in alveolar macrophages was reported (Soto et al, 2007, Carlson et al, 2008). Nevertheless, there is still limited evidence of the in vitro toxicity of AgNPs in lung cells. This clearly indicates that further research is needed to asses the impact of AgNps on human lungs.

The two most likely mechanism of AgNps toxicity is through the ROS generation and oxidative stress (Ahamed et al, 2010). When the generation of ROS reaches its anti-oxidant defense mechanism limit, oxidative stress occurs where there is a glutathione depletion and protein bound sulfhydryl groups as well as changes in various antioxidant enzymes activity which indicates lipid peroxidation have occurred (Ahamed et al, 2007). Hussain et al also reported that AgNPs act through the ROS generation and gluthathione depletion (Hussain et al, 2005, Kaur et al, 2012). There are also many reports on the induction of cytotoxicity, apoptosis and DNA damage by AgNP were arbitrated

through membrane lipid peroxidation, ROS and oxidative stress (Foldberg et al, 2009,Gopinath et al, 2010, Park et al, 2010, Wise et al, 2010). A study suggested that the increase in ROS production and the interjection of ATP synthesis which leads to DNA damage is caused by the disruption of the mitochondrial respiratory chain by AgNP (Asharani et al, 2009).

2.3 TYPES OF CELL DEATH

2.3.1 Apoptosis

2.3.1.1 Definition

TThe term ‘‘apoptosis’’ originates from the old Greek apo´pto´sisapo 'pto'sis, which means “signifies "the falling tumbling off of petals from a flower’’ blossom" or ‘‘of "of leaves from a tree in autumn’’ harvest time" which refers to the morphological appearance of the formation of “apoptotic bodies” from a cell (Kerr et al, 1972). Apoptosis is subsidiary with a change of of biochemical and physical properties including the cytoplasm, nucleus and the plasma membrane.

2.3.1.2 Morphological Alterations in Apoptosis

Generally, three major types of biochemical changes can be seen in apoptosis. First, the stimulation of caspases; second, DNA and protein breakdown and thirdly, the changes in the membrane and the identification by phagocytic cells (Kumar et al., 2010). In early apoptosis, the rounding up of cells, losing contact with neighbouring cells, and cell shrinkage. Exposition of phosphatidylserine (PS) in the outer layers of the cell membrane, which was “flipped out” from the inner layers is present. This grants early recognition of dead cells by macrophages, which results in phagocytosis without the releasing proinflammatory cellular components (Hengartner et al, 2000). In the cytoplasm, the endoplasmic reticulum widens and the cisternae bloats to form vesicles and vacuoles. Chromatin condenses and aggregates into dense compact masses in the nucleus before being disintergrated internucleosomally by endonucleases. The nucleus is then convoluted and buds off into many remnants, which are enclosed within the apoptotic bodies formed. Cell junctions are broken down in the plasma membrane, through which the plasma membrane was activated and convoluted and finally blebbs. The cell then breaks up thus causing to the several membrane spheres containing the enclosed cellular contents identified as apoptotic bodies (Kerr et al, 1994).

Under physiological conditions, some modifications in the plasma membrane takes place, enabling the phagocytic cells to recognize the

apoptotic bodies. Apoptosis mostly occurs without any leakage of cell content and inflammation since the apoptotic bodies are enclosed in an intact plasma membrane. These morphological changes are a fallout of characteristic molecular and biochemical events occurring in an apoptotic cell, most prominently by proteolytic enzymes activation. This then leads to the mediation of the cleavage of DNA into oligonucleosomal fragments in conjuction with the cleavage of a number of specific protein substrates which determine the integrity and shape of the cytoplasm or organelles (Saraste et al, 2000).

2.3.1.3 Mechanism of Apoptosis

Understanding the mechanisms of apoptosis is important as it aids in the comprehension the pathogenesis of conditions as a result of disfunctional apoptosis. This successively, may help in the evolution of drugs that aims certain apoptotic genes or pathways.

There are two main pathways involved in apoptosis; extrinsic and instrinsic pathways. Intrinsic pathway is the pathway which involves the mitochondria while extrinsic pathway is triggered by the binding of death ligand to death receptor (Masahiro Okuchi et al, 2011). Both pathways can be triggered by cytotoxic agents simultaneously or independently leading to a common pathway of apoptosis.

2.3.2 Autophagy

2.3.2.1 Pro Survival vs Pro-Death

Autophagy is a cellular lysosomal deterioration pathway which is crucial for the regulation of cell survival and death in order to retain cellular homeostasis (Levine et al, 2008, Mizushima et al, 2008). It is known that both apoptosis and autophagy has major roles in the growth, cellular homeostasis and oncogenesis of mammals. The mechanisms of apoptosis and autophagy only involve fundamentally distinct sets of regulatory and executioner molecules, thus are very much different (Danial et al, 2004, Levine et al, 2004, Mizushima et al, 2007). Autophagy can be both pro-survival or death mechanism depending on the circumstances (Mizushima et al, 2008, Levine et al, 2008). Autophagy pro-survival function is seen in various contexts including nutrient and growth factor deprivation and stress (Mizushima et al, 2008). Autophagy is also able to induce cell death possibly via the activation of apoptosis or maybe as a result of the cells inability to survive the non specific deterioration of a huge amount of cytoplasmic contents (Mizushima et al, 2008). Autophagy can also be a last choice of the route of cell death. The cross-talk between apoptosis and autophagy is thus complex and sometimes even conflicting but it is critical to the total fate of the cell (Eisenberg et al, 2009). In some cellular settings, autophagy serves as a cell survival pathway to suppress apoptosis (Yang et al, 2010). Autophagy either in collaboration with apoptosis or as a back-up mechanism could lead to cell death, when apoptosis is inhibited

(Eisenberg et al, 2009).

2.3.2.2 Mechanism of Autophagy

Mechanistic or mammalian target of rapamycin (mTOR), which is known as the major inhibitory signal that inhibits autophagy in the presence of growth factors and nutrient, is one of the key regulator of autophagy, thus the inhibition of mTOR signaling induces autophagy (Rubinsztein et al, 2007). It is a highly conserved process where the cell contents were isolated and transported through autophagosomes to lysosomes and degraded (Mukhopadhyay et all, 2014).

Studies have reported that the phosphoinositide 3-kinase (PI3K), protein kinase B (also known as AKT) and mTOR (PI3K/AKT/mTOR) are autophagy inhibitors which induce survival of cancer cells (Mirzoeva et al, 2011, Xu et al, 2011). It was found that by augmenting autophagic response, the PI3K/AKT/mTOR pathway inhibition has increased in radiosensitivity (Fujiwara et al, 2007). Besides that, the combination of dual PI3K/mTOR inhibitor NVP-BEZ235 with the mTORC1 inhibitor has proven to lead to cell death secondary to massive autophagic response (Yang et al, 2011).

2.3.2.3 PI3K-Akt Signalling Pathway

Phosphoinositol-3 kinase-protein kinase B (PI3K–AKT) signalling pathway has climbed to prominence as a key regulator of cell cycle proliferation, growth, survival, protein synthesis, and glucose metabolism. PI3K is an enzyme that phosphorylates certain components of the cell membrane. Once these components are phosphorylated, they bind to Akt.

Akt also known as protein kinase B is a serine-threonine protein kinase. Akt generally functions in the glucose metabolism, apoptosis transcription and cell migration. The well studied downstream substrate of Akt is the serine/threonine kinase mTOR (mammalian target of rapamycin). mTOR regulates the cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription (Dobashi et al, 2011).