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Ansell JE, Buttaro ML, Thomas VO (1997). Consensus guidelines for coordinated outpatient oral anticoagulation therapy management. Ann Pharmacother 31 : 604-615

Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds) Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International, pp 181-190.

Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

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Review

Novel topical drug carriers as a tool for treatment of psoriasis: Progress and advances

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Psoriasis is a chronic inflammatory skin disease portrayed by erythematous, papulosquamous lesions. It is characterized by excessive growth and aberrant differentiation of keratatinocytes. The conventional topical treatments that have been used in the past, such as coal tar and dithranol, have low efficacy, poor aesthetic and cosmetic appeal, leading to poor patient compliance while systemic therapies such as methotrexate, cyclosporine and acitretin produce significant side effects. In recent years, several novel carriers like liposomes, nanostructured lipid carriers (NLC), etc. have been used in psoriasis, with promising results. Small and relatively narrow size distribution with novel carriers permits site specific delivery to the skin, with improved drug solubilization of hydrophobic drugs and bioavailability. This review highlights the recent advancements in the field of novel carriers for topical applications of antipsoriatic active moieties and bioactives.

Key words: Novel carriers, topical, antipsoriatic drugs, psoriasis, skin.

INTRODUCTION

Psoriasis is a psychosocially, and at times medically, debilitating disorder that affect 1 to 3% of the population world wide (Afifi et al., 2005). Psoriasis has a serious impact on health related quality of life, including physical and emotional well-being (Golant and Guttman-Yassky, 2011). It basically involves excessive growth and deviant differentiation of ketatinocytes (Lowes et al., 2007). Psoriasis pathogenesis is closely associated with disease-including T helper 1 (Th1) and T helper 17 (Th17) cells (Heidenreich et al., 2009). About 80% of patients with psoriasis vulgaris are treated topically (Peeters et al., 2005). The conventional topical medications in the management of psoriasis are fraught with several limitations, which the novel carriers are reported to circumvent with safe and long term use (Witman, 2001; Prohić et al., 2007). Novel carriers such as liposome, niosome, microemulsion, nanoemulsion, nanostructured lipid carrier and ethosomes have indeed brought us closer to the goal of safe and efficacious treatment of the disease (Katare et al., 2010).

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Pathophysiology of psoriasis

Psoriasis is an immunologically mediated disease caused by activation of T lymphocytes in dermis (primarily CD4+ cells) and epidermis (predominantly CD8+ cells) (Nickoloff, 1999). The activation of T-cells is dependent upon its binding with the antigen presenting cells (APCs). This process is mediated through surface molecules used for adhesions, including leukocyte function associated antigen (LFA)-1 and CD2 on the T cells and intercellular adhesion molecule (ICAM)-1, and LFA-3 on the APCs (Lowes et al., 2004).

The T-cell receptor (TCR) for the specific T-cell recognizes an antigen presented on the major histocompatibility complex (MHC I or II) by the APC. Activated T cells proliferate and enter the circulation via trafficking through the interaction between LFA-1 and ICAM-1, and extravasate via diapedesis through the endothelium at site of inflammation in the skin. At this point, the T-cells propagate the immunologic process through the secretion of pro-inflammatory (type 1 or Th1) cytokines, which include interleukin (IL)-1, tumor necrosis

Table 1. Classification of types of psoriasis and their features (Naldi and Gambini, 2007).

S/N	Type	Features
1	Plaque	Scalp lesions are well-marginated, scaly plaques. At times severe hyperkeratosis can accumulate, tightly bound down by hair. Permanent hair loss is seldom seen, but may occur when excessive traction is applied to the hair to scale.
2	Pustular	Primarily seen in adults. It may be localized to certain areas of the body, e.g. hands and feet. Can be generalized, covering most of the body. It tends to go in a cycle as reddening of the skin followed by formation of pustules and scaling.
3	Guttate	Appears as small, red, separate spots on the skin. Lesions usually appear on the trunk and limbs and commonly number in the hundreds. Sometimes lesions form on the scalp, face and ears. They are not usually as thick as the lesions that characterize plaque psoriasis. This form can precede or co-exist with other forms of psoriasis, such as plaque.
4	Flexural	Differs from lesions elsewhere in that, although it is erythematous, it is not usually scaly. Exudation may be prominent.
5	Erythrodermic	It is the least common type of psoriasis and may occur once or more during a lifetime in 1 to 2 percent of people who develop psoriasis. It generally appears on people who have unstable plaque psoriasis. This means the lesions are not clearly defined. Widespread, fiery redness and exfoliation of the skin characterize this form. Severe itching and pain often accompanies it.

factor (TNF)-α, and interferon (IFN)-γ. These cytokines secretion results in the production of Th2 cytokine including IL-4, IL-10 and IL-11. Each cytokine down regulates the other's responses (Adorini and Trembleau, 1997). The final outcome of this bio-event is the formation of the psoriasis plaque through keratinocyte proliferation, an increase of activity and migration of other inflammatory cells and vascular changes (Hern et al., 2001).

Psoriasis has been considered basically as a disease of the keratinocyte, owing to its major clinical features of abnormal scaling and epidermal thickening. Clinically, the vascular nature of psoriasis is manifested by the erythematous plaques which exhibit pinprick bleeding on removal of scale (Auspitz sign), and is seen histologically with dilated tortuous capillaries, a prominent early feature of plaques. Histological studies have shown a fourfold increase in surface area of the superficial vascular plexus in psoriasis, and an increase in endothelial cell proliferation (Creamer, 1997). Angiogenic factors over-expressed by lesional keratinocytes include vascular endothelial growth factor (VEGF), IFN-γ, and IL-8 (IL8).

VEGF in particular, appears to play a central role in angiogenesis in psoriasis. This cytokine is also a potent mediator of inflammation and increases vascular permeability. Its synthesis by keratinocytes, activated T cells and endothelial cells, is induced by cytokines such as tumor growth factor-α (TGF-α), IFN-γ, and TNF-α. VEGF is over-expressed in psoriatic epidermis, and its receptors (KDR and Flt-1) are over-expressed on papillary microvessels. Transgenic mice over expressing VEGF in the epidermis were reported to develop a psoriasis-like condition (Xia et al., 2003). Serum concentrations of VEGF are increased in erythrodermic psoriasis (Creamer et al., 1996), and a correlation between plaques VEGF, psoriasis area and severity index (PASI) and serum VEGF values was also reported (Creamer et al., 2002). The hyperpermeability induced by VEGF has been proposed as the cause of microalbuminuria and pulmonary edema in patients with severe psoriasis.

The main factors responsible for psoriasis therefore include the angiogenic factors, over-expression of VEGF in the psoriatic epidermis, transfer in bone marrow transplants from affected individuals, reduced TNF concentrations and increased concentrations of natural killer (NK T) cells.

Psoriasis has been classified into several types as presented in Table 1. Table 2 lists the various drugs that have been used for psoriasis.

Need for novel carriers

Presently, it has become increasingly apparent that the development of new drug alone is not sufficient to ensure progress and success in drug therapy, since the main reasons for the failure of therapy remain poor drug solubility, insufficient drug concentration due to poor absorption, rapid metabolism and elimination, drug distribution to other tissues combined with high drug toxicity, and high fluctuation of plasma level due to unpredictable bioavailability after per oral administration, including the influence of food on plasma levels (Mehnert and Mader, 2012). A promising strategy to overcome

Table 2. Various drugs for psoriasis with their mechanism of action and side effects.

Compound	Mechanism of action	Side effect	Reference
Coal tar	DNA suppression	Messiness, staining, odor, irritant, allergic, phototoxic responses and folliculitis	Walter et al. (1978)
Psoralens	Suppresses DNA synthesis by cross-linking DNA strands	Long term risks include skin cancer and premature ageing of skin	Greaves et al. (1978)
5-Aminolevulinic acid	Antiproliferative effect	Photosensitivity and irritation	Ibbotson (2002)
Anthralin	Antiproliferative effect	Irritation and staining of the skin, nails and clothing	Fuchs et al. (1990)
Tazarotene	Antiproliferative and anti-inflammatory activities	Pruritis, erythema, burning and desquamation	Weinstein et al. (2003)
Vitamin D analogues	Keratinocyte proliferation, enhance cellular differentiation	Irritation and hypercalcemia	Takahashi et al. (2003)
Acitretin	Normalizes keratinocytes proliferation and differentiation	Mucocutaneous reactions, pruritus and hypertriglyceridemia	Rossi and Pellegrino (2009)
Corticosteroids	Anti-inflammatory and immunosuppressant	Burning, skin atrophy, photosensitivity and irritation	Lebwohl et al. (2005)
Methotrexate	Anti-inflammatory and immunosuppressant	Myelosuppression, hepatic fibrosis and pulmonary fibrosis	Leon et al. (2007)
Cyclosporine	Immunosuppressant	The risk of infection, with long term use and non-melanoma skin cancer	Heydendael et al. (2003)
Tacrolimus	Immunosuppressant	Burning, itching, skin cancer and lymphoma	Scheinfeld (2004)
Pimecrolimus	Immunosuppressant	Burning, itching, skin cancer and lymphoma	Scheinfeld (2004)

these problems includes development of suitable drug carrier system to achieve controlled and localized delivery of the active drug according to the specific need of the therapy. The size of the carriers designed depends on the desired route of administration, and ranges from few nanometers (colloidal carriers) to the micrometer range (microparticles), and to several millimeters (implants). Implants and microparticles are too large for drug targeting and intravenous administration (Nanjwade and Patel, 2011). Therefore, colloidal carriers have attracted increasing attention during the recent years. Investigated systems include nanoparticles, nanoemulsions, microemulsions, liposomes, ethosomes, nanosuspensions, micelles and soluble polymer-drug conjugates.

Topical drug carriers and challenges in dermal delivery

During the last decades, inorganic and colloidal particles such as nanocapsules, nanospheres, nanostructured lipid carrier, etc. have been explored for dermal/transdermal drug delivery. The successful implementation of these systems for drug delivery completely depends on their ability to penetrate through several anatomical barriers, sustained release of their content and their stability in the nanometer size. Different colloidal drug delivery systems (including liposomes, dendrimers and polymeric nanoparticles, in addition to solid lipid nanoparticles), were developed to overcome physicochemical limitations of potential therapeutic compounds such as poor solubility, low permeability, short half-life, high molecular weight, side effects and systemic toxicity (Almeida and Souto, 2007).

Stratum corneum is the main barrier in the percutaneous absorption of topically applied drugs. Small and relatively narrow size distribution with novel carrier permit site specific delivery to the skin with improved drug solubilization of hydrophobic drugs and better bioavailability. The problem of lipid imbalance can be resolved by delivering the unsaturated fatty acids like linoleic acid to restore the normal skin conditions by lipid carriers (Morganti et al., 2001). This provides lipid enriched hydrating conditions to help retain the drug molecules within the dermal layers at or near to the site of action.

NOVEL CARRIERS USED IN PSORIASIS

In recent years, attempts have been made for successful delivery of antipsoriatic agents by employing novel carriers (Table 3).

Liposome

Liposomes are microscopic structures consisting of one or more concentric spheres of lipid bilayers enclosing aqueous compartments. As drug carrier

systems for topical treatment, liposomes are reported to be superior over conventional topical preparations. Phospholipids, being the major component of liposomal systems, are easily integrated with the skin lipids and maintain the desired hydration conditions to improve drug penetration and localization in the skin layers (Moghimi and Patel, 1993; Cevc et al., 1996; Schmid and Korting, 1996).

The use of liposomes as drug carriers seems to be promising, exhibiting therapeutic prospects with a reduction of side effects. Liposomal encapsulation of retenoids, for example vitamin A acid or tretinoin reduced the local irritation (Trapasso et al., 2009). Reports suggest that liposomal encapsulation of tacrolimus in topical formulation enhanced its penetration through skin. These characteristics may allow liposomal tacrolimus to be effective against psoriasis without the need of occlusive dressings. If liposomal tacrolimus penetrate into the blood stream, reports with other models suggest that it will be less toxic than free tacrolimus. This will be more important to patients who require higher concentrations of ointment to large areas of skin (Patel et al., 2010). Bhatia et al. (2004) developed and characterized tamoxifen loaded liposome for topical therapy and reported improved skin permeation of Tamoxifen (TAM) and retention in the skin.

Calcipotriol, a vitamin D analogue was successfully delivered in lipopolymer poly(ethylene glycol)-distearoylphosphoethanolamine (PEG-DSPE) liposomes with a significant increase in drug deposition into the stratum corneum (Knudsen et al., 2012). The size of the liposomes affected the penetration of calcipotriol into the stratum corneum, as smaller unilamellar vesicles enhanced drug penetration as compared to large multilamellar vesicles. This also indicated that the liposomes to some extent migrate as intact vesicles into the stratum corneum. Srisuk et al. (2012) compared the physicochemical characteristics and *in vitro* permeability of methotrexate entrapped deformable liposomes prepared from phosphatidylcholine (PC) and oleic acid, with conventional liposomes prepared from PC and cholesterol (CH). The deformable liposomes displayed enhanced permeability and this was attributed to the elastic characteristics of the oleic acid containing liposomes, as well as skin penetration enhancer effect of oleic acid.

Nagle et al. (2011) investigated the potential benefits of combining menthol with methotrexate in a vesicular gel base for improving the drug penetration, its dermal availability and patient acceptability. Antipsoriatic efficacy of the formulations tested *in vivo*, using the rat tail model, indicated that the vesicular gel containing menthol led to maximum drug retention in the skin. The *in vivo* studies also ascertained the effectiveness of the formulation in inducing a normal pattern of differentiation in the rat tail skin that initially showed parakeratosis, which is also characteristic of psoriatic epidermis.

Li et al. (2010) reported an ultra deformable cationic liposome for transdermally delivering plasmid DNA to mouse skin. The antipsoriatic efficacy in the K14-VEGF transgenic mouse model by transdermal delivery of murine IL-4 was observed. Plasmid DNA was transdermally delivered to vicinal sites of epidermis and hair follicles. Plasmid DNA expression was detected in ear skin.

Niosome

The low cost, greater stability and ease of storage of non-ionic surfactants led to the exploitation of these compounds as alternative to phospholipids, the main constituent of liposomes (Uchegbu and Vyas, 1998). Niosomes are microscopic lamellar structures formed on admixture of a non ionic surfactant, cholesterol and a charge inducing agent, with subsequent hydration in aqueous media (Uchegbu and Florence, 1995; D'Souza et al., 1997). Niosomes comprises of an architecture consisting of both hydrophobic and hydrophilic moieties, and as a result can accommodate drug molecules of varying solubilities (Namdeo and Jain, 1996). Niosomes have been evaluated in many pharmaceutical applications and were reported to reduce systemic toxicity by drug encapsulation and minimize clearance of such agents from the body by slow drug release. Agarwal et al. (2001) developed dithranol entrapped in liposomal and niosomal vesicles (0.5%), and found both of them superior to conventional formulation, while liposomes showed better results than niosomes employing mice skin. Marianecci et al. (2012) investigated the niosomes made up of surfactants (Tween 85 and Span 20) and cholesterol for the delivery of ammonium glycyrrhizinate (AG), useful for the treatment of various inflammatory based diseases. The *in vitro/vivo* efficacy of the ammoniu glycyrrhizinate/niosomes was studied in murine and human models of inflammation. The AG-loaded non-ionic surfactant vesicles showed no toxicity, good skin tolerability and were able to improve the drug anti-inflammatory activity in mice. Furthermore, an improvement of the anti-inflammatory activity of the niosome delivered drug was observed on chemically induced skin erythema in humans.

Niosomal methotrexate in chitosan gel was tested for irritation and sensitization on healthy human volunteers, followed by its assessment through double-blind placebo-controlled study on psoriasis patients (Lakshmi et al., 2007). The human repeated insult patch test did not produce any significant irritation or sensitization on healthy human volunteers. The placebo and marketed gels were compared with niosomal methotrexate gel. At week 12, with niosomal methotrexate gel, there was reduction in total score from 6.2378 ± 1.4857 to 2.0023 ± 0.1371. These results suggested better efficacy of niosomal methotrexate gel as compared to placebo and marketed methotrexate gel.

Table 3. Previous work on novel carriers for psoriasis.

Drug	Limitations of conventional form	Novel carrier	Effect observed	Reference
Tacrolimus	Normal skin transport	Liposome	Facilitated entry into the tough barrier consisting of stratum corneum. Better solubility of drug	Patel et al. (2010)
		Nanolipid carrier	Provide good occlusion property and solubility	Pople and Singh (2011)
Coal Tar	Skin irritation and staining	Phospholipid lipid carrier	Reduce irritation and better solubility	Bhatia et al. (2008)
Anthralin	Irritation and staining	Liposome	Increase the stability of drug. Increases the penetration of drug through skin	Saraswat et al. (2007)
		Niosome	Enhance stability of drug	Agrawal et al. (2001)
		Liposome	Increases drug uptake and target deeper skin layer	Mahrle et al. (1991)
Vitamin D analogues	Local irritation	Nanostructured lipid carrier	Enhanced skin permeation and negligible skin irritation	Lin et al. (2010)
Corticosteroids	Skin irritation	Nanocapsule suspension	Greater stability of drug	Fontana et al. (2010)
		PEG-NLCs	High entrapment efficiency and improved drug stability	Doktorovova et al. (2010)
5-aminolevulinic acid	Poor skin penetration	Liposome	Enhance drug uptake	Casas and Batlle (2006)
		Liposome	Compatible with skin lipid	Venosa et al. (2008)
		Ethosome	Better skin permeation and targeting deeper skin layer	Fang et al. (2009)
		Nanoemulsion	optimize topical drug permeation negligible skin disruption and acceptable safety	Zhang et al. (2011)
Temoporfin	Low aq. solubility	Liposome	Higher rate of drug transfer across skin	Dragicevic-Curic et al. (2009a)
		Invasome	Enhanced drug uptake	Dragicevic-Curic et al. (2009b)
Psoralens	Low penetration	Microemulsion	Enhanced drug uptake and provide photostability	Baroli et al. (2000)
Methotrexate	Low penetration	Liposome hydrogel	Enhance drug uptake	Ali et al. (2008)
		Deformable liposome	Better skin permeation and targeting deeper skin layer	Trotta et al. (2004)
		Niosome	Enhance stability of drug	Lakshmi et al. (2007)
		Microemulsion	Enhanced drug uptake and provide photostability	Zhi and Jin (2011)
		Nanogel	Greater stability of drug	Ali et al. (2008)
Cyclosporine	Limited cutaneous permeation	Microemulsion	Increase the stability of drug	Umezawa and Ozawa (2007)
		PLGA-nanoparticle	Rapid dissolution of drug and good stability	Jain et al. (2011)
Acitretin	Scaling, erythema, burning, stinging	Nanostructured lipid carrier	No major systemic side effects, the main topical side effect was irritation	Agrawal et al. (2010)

Microemulsion

Microemulsions are thermodynamically stable isotropic systems in which two immiscible liquids (water and oil) are mixed to form a single phase by means of an appropriate surfactant or its mixture. The short to medium chain alcohols are generally considered as co-surfactants in the microemulsion system. The presence of surfactant and co-surfactant in the system makes the interfacial tension very low. Therefore, microemulsions form spontaneously, with an average droplet diameter of 10 to 140 nm. Microemulsions have the ability to deliver larger amounts of water and topically applied agents into the skin than water alone or other traditional vehicles such as lotions or creams because they act as a better reservoir for a poorly soluble drug through their solubilization capacity. Although microemulsion can be used to deliver drugs via several routes, the system has been extensively

studied as a vehicle for topical and transdermal administration (Peltola et al., 2003; Sintov and Botner, 2006; Kreilgaard, 2002).

In topical and transdermal formulations, microemulsions have increased the cutaneous absorption of both lipophilic and hydrophilic APIs when compared to conventional vehicles (emulsions, pure oils, aqueous solutions, etc). Due to their special features, microemulsions offer several advantages for pharmaceutical use, such as ease of preparation, long-term stability, high solubilization capacity for hydrophilic and lipophilic drugs, and improved drug delivery (Karasulu, 2008; Heusehkel et al., 2008).

Zhi and Jin (2011) prepared methotrexate loaded microemulsion and evaluated microemulsion quality and percutaneous penetration through the skin. Umezawa and Ozawa (2007) reported that cyclosporine microemulsion increased the percutaneous absorption in skin of patients with psoriasis. Baroli et al. (2000) reported that microemulsion for topical delivery of 8-methoxsalen increased the photostability and enhanced the percutaneous absorption of drug. Alvarez-Figueroa and Blanco-Mendez (2001) investigated the effectiveness of transdermal administration of methotrexate (MTX) by iontophoretic delivery from two types of hydrogel and passive delivery from two types of microemulsion by *in vitro* assays. Both iontophoretic delivery of MTX from hydrogels and passive delivery from microemulsions were more effective than passive delivery from aqueous solutions of the drug. In the passive delivery assays, both water/oil (w/o) and oil/water (o/w) microemulsions were used, and the effectiveness of delivery from o/w systems was higher. At the end of all assays, significant amounts of MTX were detected in the skin.

Raza et al. (2011) developed and characterised a novel dithranol-containing phospholipid microemulsion for enhanced skin permeation and retention. The microemulsion systems composed of isopropyl myristate and Tween 80, with mean particle diameter of 72.8nm showing maximum skin permeation (82.23%), skin permeation flux (0.281mg/cm²/h) along with skin retention (8.31%) with regard to systems containing tocopherol acetate and Tween 20.

Nanoemulsion and nanoemulsomes

Nanoemulsions are obtained when the dimension of an emulsion globule reaches approximately 20 to 500 nm. The small droplet size can resist the physical destabilization caused by gravitational separation, flocculation and/or coalescence. It also avoids the creaming process because the droplet's Brownian motion is enough to overcome the gravitational separation force. The size and polydispersity of nanoemulsions can affect properties such as particle stability, rheology, appearance, color, texture and shelf life. They are not formed spontaneously, and their properties depend on the thermodynamic conditions and preparation methods. Nano/submicron emulsions are well accepted for their ability to increase skin permeation, prolonged action on the skin, and protection of the drug from instability (Solans et al., 2005; Weyenberg et al., 2007).Zhang et al. (2011) formulated 5-aminolevulinic acid (ALA) and methyl 5-aminolevulinic acid (mALA) at equimolar concentrations in o/w and w/o nanoemulsions for skin delivery and showed that apart from drug, the emulsion type and the oil phase are all important considerations when attempting to optimize topical drug permeation. The o/w soybean oil dispersions were most promising because of their ability to exert the highest *in vitro* ALA flux. The uniformity of drug flux by the emulsions was improved when compared to the aqueous control. The addition of α-terpineol, a penetration enhancer, as a part of the oil phase did not further increase drug permeation via the skin.Khandavilli and Panchagnula (2007) formulated a nanoemulsion (NE) to achieve penetration of paclitaxel into deeper skin layers while minimizing the systemic escape. Upon dermal application, the drug was predominantly localized in deeper skin layers, with minimal systemic escape. Bernardi et al. (2011) formulated rice bran oil nanoemulsions and evaluated it for irritation potential and moisturising activity on volunteers with normal and diseased skin types.

Optimized dithranol-loaded emulsomes were found to substantially enhance the antipsoriatic activity on a mouse-tail model with regard to marketed product (Raza et al., 2012). Also, the selected composition enhanced drug permeation and marked skin retention. The formulation was found to be quite non-irritant, stable and biocompatible in comparison to the marketed product.

Nanostructured lipid carrier

Nanostructured lipid carriers (NLCs) are the latest generation of Solid lipid nanoparticles (SLNs) possessing improved properties of drug loading, modulation of the release profile, and stable drug incorporation during storage. NLCs are produced by mixing solid lipids with spatially incompatible lipids leading to a lipid matrix with a special structure. A blend of a liquid and solid lipid creates a less perfect crystalline structure with many imperfections, providing more space for drug accommodation. Examples of solid lipids include triglycerides (tristearine, tripalmitine, trimyristine), fatty acids (stearic acid, palmitic acid), waxes (carnauba, cetyl palmitate), whereas liquid lipids include medium chain triglycerides, oleic acid, isopropyl myristate. Their suitability for dermatological applications was also confirmed by successful formulations of drugs for treatment of skin diseases. NLC systems are a promising carrier for the topical delivery of antipsoriatic drugs as revealed by enhanced skin permeation, negligible skin irritation, and the compatibility of the drugs (Muller et al., 2007).

Lin et al. (2010) combined calcipotriol and methotrexate in nanostructured lipid carriers for topical delivery, and reported efficient delivery of the drugs. Nam et al. (2007) investigated tacrolimus loaded nanostructured lipid carriers for topical delivery. The penetration rate of these NLCs through the skin of a hairless mouse was greater than a commercial dermal ointment containing tacrolimus. Fluticasone propionate in novel polyethylene glycol (PEG)-containing nanostructured lipid carriers in the presence of PEG in NLC composition improved the physicochemical stability of the developed NLC (Doktorovova et al., 2010).

Ethosome

Ethosomes are noninvasive delivery carriers that enable drugs to reach the deep skin layers and/or the systemic circulation. These are soft, malleable vesicles tailored for enhanced delivery of active agents. They are composed mainly of phospholipids, (phosphatidylcholine, phosphatidylserine, phosphatidic acid), high concentration of ethanol and water. It is reported that ethosomes penetrate the skin and allow enhanced delivery of various compounds to the deep strata of the skin or to the systemic circulation (Touitou, 2002).

The high concentration of ethanol in ethosomes causes disturbance of skin lipid bilayer organization, hence when incorporated into a vesicle membrane, it enhances the vesicle's ability to penetrate the stratum corneum. Also, because of their high ethanol concentration, the lipid membrane is packed less tightly than conventional vesicles but has equivalent stability, allowing a more malleable structure and improves drug distribution ability in stratum corneum lipids (Merdan et al., 1998). Trotta et al. (2004) prepared deformable liposomes for dermal administration of methotrexate, and these provided maximum dose administration through the skin that may be of value for topical administration of methotrexate in the treatment of psoriasis.

Novasome

Novasomes are the modified forms of liposomes or a variation of niosomes prepared from the mixture of monoester of polyoxyethylene fatty acids, cholesterol and free fatty acids at 74/22/4 ratio and are 0.1 to 1.0 µm in diameter.

They consist of two to seven layered shells that surround an unstructured space occupied by a large amorphous core of hydrophilic or any water-soluble molecules that have been mixed with the hydrophobic materials (Singh et al., 2011). An agonist of parathyroid hormone related peptide receptor, PTH (1-34), was formulated in Novasome A cream (Holick et al., 2003). Novasome A cream enhanced the percutaneous absorption of PTH (1-34) in human skin in comparison with formulations in propylene glycol or normal saline. Psoriatic lesions treated with PTH (1-34) showed marked improvement in scaling, erythema and induration.

Solid lipid nanoparticle

During the last two decades, numerous reports have described various SLN formulations that may find applications in drug delivery. The SLN structure is composed of solid core, which may contain triglycerides, glyceride mixtures, or waxes that are solid at both room temperature and human body temperature. SLNs are interesting lipid based drug delivery carriers for a number of reasons, including their nanoscale particle size and their biocompatible and biodegradable components. Urban-Morlan et al. (2010) prepared and characterized solid lipid nanoparticle containing cyclosporine A (CyA) by emulsification-diffusion method. Gambhire et al. (2011) reported the preparation and optimization of dithranol loaded solid lipid nanoparticles. The results of the *ex vivo* penetration studies demonstrated about two-fold increase in localization of delayed type IV hypersensitivity (DTH) in skin with SLN entrapped ointment compared to plain DTH.

HERBAL DRUG USED FOR PSORIASIS

World Health Organization (WHO) notes that 74% of the plant derived medicines are used in modern medicine in a way that their modern application directly correlates with their traditional use as herbal medicines by native cultures (Kumar et al., *2003*). WHO estimated that 65 to 80% of the world's population uses traditional medicines as their primary form of health care, and about 85% of traditional medicines involve the use of herbal preparations.

Various bioactives and phytoconstituents have been delivered using novel carriers in psoriasis as displayed in Table 4.

CONCLUSION

Despite the significant advances that have been made in understanding the pathomechanisms of psoriasis and in identifying effective treatment approaches, the search for optimum treatment strategies for psoriasis still remains a major challenge.

Numerous topical treatment modalities are available for psoriasis, but there is a dearth of uniformly acceptable approach for moderate-to-severe psoriasis. A variety of lipid based novel drug delivery systems like lipid nanoparticles, microemulsion, nanoemulsions, nanoemulsomes,

Table 4. Bioactives delivered through novel carriers for psoriasis.

Bioactives	Novel carrier	Effect observed	Reference
Psoralea carylifolia (babchi oil)	Microemulsion gel	Psoralea corylifolia seed oil was effective with improved stability	Kumar and Parmar (2003)
Curcuminoids	Nanoparticles	Enhanced skin uptake and better retention	Mulik et al. (2009)
Glycyrrhetic acid	Nanoemulsion	Increased the stability of formulation and better transdermal effect	Puglia et al. (2010)
Tea tree oil	Microemulsion	Potential in enhanced drug uptake	Khokhra and Diwan (2011)
Cholchicine	Liposome	Sustained delivery	Singh et al. (2009)
3,5-dihydroxy-4-isopropylstilbene	Nanoemulsion	Dramatically improved the DHPS transdermal effect, and the osmotic quantity of DHPS from the nanoemulsion formulations	Zhang et al., 2011

liposomes, niosomes and nanostructured lipid carriers clearly present a tool to overcome the many challenges associated with topical antipsoriatic drug therapy.

These novel carriers have been widely studied and have demonstrated an increment in the rate and extent of drug delivery and optimal therapeutic outcomes. Thus, there is need for further investigations with antipsoriatic drugs to establish the clinical utility and industrial scale-up of techniques for manufacturing these potential novel carriers.

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Review

Antimalarial drugs: Mode of action and status of resistance

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Malaria is a major global health problem, with an estimated 300 to 500 million clinical cases occurring annually. Malaria remains one of the leading causes of disease and death in the tropics, mainly of children under 5 years of age. The most prevalent and dangerous type of malaria is caused by *Plasmodium falciparum. P. vivax* is a common cause of malaria in Latin America, Asia, and Oceania, but not Africa. *P. malariae* and *P. ovale* are much less common. Antimalarials are used in three different ways: prophylaxis, treatment of falciparum malaria, and treatment of non-falciparum malaria. Prophylactic antimalarials are used almost exclusively by travelers from developed countries who are visiting malaria-endemic countries. The antimalarials in common use come from the following classes of compounds: the quinolines (chloroquine, quinine, mefloquine, amodiaquine, primaquine), the antifolates (pyrimethamine, proguanil and sulfadoxine), the artemisinin derivatives (artemisinin, artesunate, artemether, arteether) and hydroxynaphthaquinones (atovaquine).

Key words: Malaria, resistance, antimalarial drugs, plasmodium.

INTRODUCTION

Malaria is caused by infection with a single-cell parasite, *Plasmodium*. Four *Plasmodium* spp. cause malaria in human beings: *Plasmodium falciparum*, *P. vivax, P. ovale,* and *P. malariae. P. falciparum* is the most important because it accounts for the majority of infections and causes the most severe symptoms. Malaria remains one of the leading causes of morbidity and mortality in the tropics. According to the World Malaria Report (2011), there were 106 malaria endemic countries in 2010.

There were 216 million cases of malaria in 2010; 81% of these were in the World Health Organization (WHO) African region. An estimated 3.3 billion people are at risk of malaria. An estimated 655, 000 persons died of malaria in 2010. 86% of the victims were children under 5 years of age, and 91% of malaria deaths occurred in the African region. Antimalarials are used in three different ways: prophylaxis, treatment of falciparum malaria, and treatment of non-falciparum malaria. Prophylactic antimalarials are used almost exclusively by travelers from developed countries who are visiting malaria-endemic countries. Treatment protocols for falciparum malaria vary, depending on the severity of the disease; fast-acting, parenteral drugs are best for severe, life-threatening disease. In addition, treatment protocols for falciparum malaria vary geographically and depend on the resistance profiles for strains in particular regions. Non-falciparum malarias, in contrast, rarely are drug-resistant. In addition, *P. vivax* and *P. ovale* have dormant liver stages that can cause relapses months to years after an infection is cleared, so they need to be treated with an additional agent that can clear this stage. The antimalarials in common use come from following classes of compounds: the quinolines (chloroquine, quinine, mefloquine, amodiaquine, primaquine), the antifolates (pyrimethamine, proguanil and sulfadoxine),the artmisinin

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derivatives (artemisinin, artesunate, artemether, arteether) and hydroxynaphthaquinones (atovaquine). This review looks at the drugs in common use and their treatment regimens, pharmacokinetic properties, mechanism of action and resistance, as well as status of resistance.

CHLOROQUINE

Chloroquine was first synthesized in Germany, but it was not recognized as a potent antimalarial drug until the 1940s during the US World War II military effort. By 1946, it was found to be far superior to other contemporary synthetic antimalarials (Coggeshall and Craige, 1949). Chloroquine became the cornerstone of antimalarial chemotherapy for the next 40 years. It quickly became the drug of choice globally to treat uncomplicated *P. falciparum* infections, and it was used as part of the Global Malaria Eradication campaign launched by the WHO in 1955. Chloroquine is one of the least expensive antimalarials available and is still in widespread use. This drug can be taken both as a prophylactic and as a treatment.

Despite much research during the last 40 years, the exact mechanism by which chloroquine kills the malaria parasite remains controversial (Foley and Tilly, 1997; Foote and Cowman, 1994; Peters, 1997). This drug inhibits DNA and RNA biosynthesis and induces the rapid degradation of ribosomes and the dissimilation of ribosomal RNA. The inhibition of protein synthesis is also observed evidently as a secondary effect. It has been proposed that the inhibition of DNA replication is the general antimicrobial mechanism of action of chloroquine. Chloroquine accumulates in very high concentrations in the parasite food vacuole (Geary et al., 1986). Once in the food vacuole, chloroquine is thought to inhibit the detoxification of heme. Chloroquine becomes protonated (to CQ^{2+}) because the digestive vacuole is acidic (pH 4.7) and subsequently cannot leave the vacuole by diffusion. Chloroquine caps hemozoin molecules and prevents the further biocrystallization of heme, thus leading to heme buildup. Chloroquine binds to heme (or FP) to form what is known as the FP-chloroquine complex; this complex is highly toxic to the cell and disrupts membrane function. The actions of the toxic FP-chloroquine complex and FP result in cell lysis and ultimately the auto-digestion of the parasite cell. In essence, the parasite cell drowns in its own metabolic products.

Mechanism of resistance

Resistance to chloroquine was slow to develop, taking almost 20 years, despite extensive use of the drug, suggesting that mutations in several genes were required to produce the resistance phenotype. The mechanism of chloroquine resistance also is uncertain. Chloroquine-resistant parasites accumulate less chloroquine in the food vacuole than do sensitive parasites (Fitch, 1970) and one assumption is that chloroquine resistance is not based on the mode of action of the drug but on the access of the drug to the parasite food vacuole. Early studies indicated that chloroquine resistance was associated with an elevated level of drug efflux. Drug-resistant parasites were reported to release pre-accumulated chloroquine almost 50 times faster than chloroquine-sensitive isolates (Verdier et al., 1985; Krogstad et al., 1987). Furthermore, verapamil was shown to reduce the apparent rate of drug efflux from chloroquine-resistant parasites (Krogstad et al., 1987). Since verapamil is known to reverse the P-glycoprotein-mediated efflux of drugs in multidrug-resistant tumor cells (Martin et al., 1987), this led to the proposal that efflux of chloroquine by a plasmodial P-glycoprotein is responsible for chloroquine resistance.

Bray et al. (1999) on the other hand have suggested that chloroquine resistance is caused by reduced affinity of chloroquine for heme, thereby reducing chloroquine uptake. Another proposal is that chloroquine is transported actively through the parasite by the Na^+/H^+ exchanger (NHE) and that resistance to chloroquine is mediated by mutations in the NHE (Wunsch et al., 1998) but this suggestion has been disputed. Wellems et al. (1990, 1991) analyzed a cross between a chloroquine-resistant and a chloroquine-sensitive strain of *P. falciparum*, and identified a chloroquine-resistance locus within a 400 kb segment of chromosome 7. Su et al. (1997) mapped the putative chloroquine-resistance locus to a 36 kb region and identified the open reading frames of 8 potential genes within this region. Initially, chloroquine resistance was thought to be caused by *cg2,* a gene coding for a polymorphic protein located at the parasite periphery. However, recent transformation studies have ruled out *cg2* and suggest another gene, *pftcr* within this region (Fidock et al., 1999).

Status of resistance

Chloroquine, soon after introduction in the 1950s, quickly became the main drug of choice globally to treat uncomplicated *P. falciparum* infections, for instance, as part of the Global Malaria Eradication Campaign launched by the (WHO) in 1955. However, *P. falciparum* did eventually develop resistance to chloroquine, and has spread to almost all the endemic countries today (Wellems and Plowe, 2001). Chloroquine resistant parasites in Africa were thought by some to share the same origin as the Indo-China strains, but by others to have developed locally as a result of mass drug administration plus intrinsic entomological, epidemiological, and parasitological factors that promoted local resistance selection (Diribe and Warhurst, 1985).

Current molecular studies suggest the Asian origin of African isolates, but at least four different foci of chloroquine resistance have so far been identified (Warhurst, 1995). Resistance to chloroquine has spread to almost all the countries thus limiting the effective use of this low cost antimalarial.

QUININE

Quinine is derived from the bark of the cinchona tree and was used for treating fevers as early as the 17th century, although not until 1820, it was the active ingredient of the bark, isolated and used in its purified form. Quinine is used as a treatment for uncomplicated and severe malaria in many different therapeutic regimens. Quinimax, which is a combination of quinine, quinidine, and cinchonine (all derived from cinchona bark), is also used (Deloron et al., 1985). Quinine must be administered for at least 7 days to non-immune populations (Bunnag and Harinasuta, 1986; Krishna and White, 1996; Bjorkman et al., 1991) but it is effective in immune populations (such as in sub-Saharan Africa) when given for 3 to 5 days because it appears to be potentiated by the host immune system (Miller et al., 1989). In the United States where quinine is not available commercially, quinidine (its D-isomer) is used. Quinine is also used in combination with antibiotics (tetracycline or doxycycline).

Mode of action

Quinine acts in a manner similar to that of chloroquine but with some differences; chloroquine causes clumping of the malaria pigment, whereas quinine antagonizes this process (Peters, 1987). In addition, quinine is a weaker base than chloroquine and has less affinity for heme, implying that mechanisms other than ion transport into the food vacuole and heme-drug interactions are required for the action of these drugs (Foley and Tilly, 1998). Quinine also interacts rather weakly with heme (Kd = 2.63×10^{-6} M) (Chou et al., 1980), but has been shown to inhibit heme polymerization (Slater 1992; Chou and Fitch, 1993) and heme catalase activity (Ribeiro et al., 1997). In the absence of a specific transporter, quinine is likely to be accumulated less efficiently in the food vacuole than chloroquine. Further work is required to determine whether the mechanism of action of quinine is more closely aligned to that of chloroquine.

Status of resistance

Although quinine treatment failure has been reported, many of these instances can be attributed to inadequate treatment. Reports of clinical quinine resistance have been sporadic, with the highest incidence occurring in Southeast Asia, where it was first reported in 1967 (WHO, 1967). In Thailand, treatment failure rates increased from 6% in 1978 to 14% in 1979 to 1980, and they were up to 38.5% in 1981 (Chongsuphajaisiddhi et al., 1983). A more recent report shows 23% recrudescence in pregnant women after quinine treatment (McGready et al., 1998), which may be an indication that quinine resistance in Thailand is stabilizing, perhaps because of the widespread use of quinine combinations and alternative drugs. In Africa, quinine resistance remains at very low levels, and even in Southeast Asia, cure rates with quinine combinations (for example, quinine-tetracycline) remain high (Watt et al., 1992; Looareesuwan et al., 1994; Bunnag et al., 1996).

MEFLOQUINE AND HALOFANTRINE

Mefloquine was developed in the 1970s by the United States Army in response to the increasingly poor cure rates of chloroquine, with clinical trials beginning in 1972 (Davidson et al., 1975; Trenholme et al., 1975; Rieckmann et al., 1974). Mefloquine has a very long half-life both in patients with malaria (10.3 to 20.5 days) (Karbwang and White, 1990; Na-Bangchang et al., 1995) and in healthy volunteers (13.8 to 27.5 days). Mefloquine is recommended for prophylaxis and therapy in chloroquine-resistant areas. Despite considerable publicity about possible neuropsychiatric side-effects of mefloquine, the same evidence is not conclusive (Choo, 1996).

Halofantrine is a tricyclic compound that was developed at approximately the same time as was mefloquine and has been used as a second-line agent; its use may be limited by its cardiotoxic side-effects and variable pharmacokinetics.

Mode of action

Mefloquine interacts relatively weakly with free heme, with reported Kd values ranging from 3.3×10^{-7} to 1.63×10^{-5} M (Chou et al., 1980; Chevli and Fitch, 1982). Mefloquine has been shown to inhibit heme polymerization *in vitro* with a similar (Slater and Cerami, 1992; Slater, 1993) or lower (Chou and Fitch, 1993; Raynes et al., 1996) efficiency than chloroquine (that is, close to millimolar levels). Given the lower basicity of mefloquine, it seems unlikely that it would reach the intravacuolar concentration required to inhibit heme polymerization. Furthermore, while chloroquine treatment of *P. berghei* infected mice was found to cause a decrease in hemozoin production, mefloquine and quinine had no effect (Chou and Fitch, 1993). Mefloquine

is also a much less potent enhancer of the peroxidase activity of heme than chloroquine (Sugioka and Suzuki, 1991) and has been shown to interfere with the ability of chloroquine to enhance heme-induced cell lysis (Dutta and Fitch, 1983).

The available data suggest therefore that, mefloquine interferes with a different step in the parasite-feeding process than chloroquine (Geary et al., 1986). Desneves et al. (1996) used the technique of photo affinity labeling to identify two high-affinity, mefloquine-binding proteins with apparent molecular masses of 22 to 23 kDa and 36 kDa in *P. falciparum* infected erythrocytes. The identities of these polypeptides have not been established yet, but they may be involved in mefloquine uptake or action. There is also increasing evidence to suggest a role for the plasmodial P-glycoprotein (P-glycoprotein homolog-1, Pgh-1) in mefloquine resistance. This raises the possibility that Pgh-1 may also be the target of action of mefloquine.

Mechanism of resistance

Mefloquine resistance in field isolates of *P. falciparum* is associated with amplification of the *pfmdr1* gene (Peel et al., 1993; Wilson et al., 1993; Cowman et al., 1994) and over-expression of its protein product Pgh-1 (Cowman et al., 1994). Moreover, selection for mefloquine resistance *in vitro* leads to amplification and over expression of the *pfmdr1* gene (Cowman et al., 1994; Wilson et al., 1989; Peel et al., 1994). This has led to the idea that Pgh-1 is responsible for at least some forms of mefloquine resistance. Resistance to halofantrine and quinine also increased during mefloquine selection, suggesting a similar underlying mechanism (Peel et al., 1993, 1994; Cowman et al., 1994). Resistance to mefloquine is not reversed by verapamil or chlorpromazine, but can be reversed by penfluridol (Peters and Robinson, 1991).

Status of resistance

Resistance to mefloquine has been rising inexorably ever since this drug was introduced in the 1970s (Boudreau et al., 1982; Espinal et al., 1985; Draper et al., 1985; Brasseur et al., 1990; Gay et al., 1990; Raccurt et al., 1991; Looareesuwan et al., 1992; White, 1994). In an area of Thailand where mefloquine was used intensively, substantial mefloquine resistance developed within 5 years of its introduction (White, 1994; Mockenhaupt, 1995). Cure rates with mefloquine have now dropped to 41% in some areas of Thailand (Nosten et al., 1991; Fontanet et al., 1993). Combination regimens with either artenusate or artemether have been introduced in an effort to stem the development of resistance to mefloquine (Price et al., 1995). There have been reports of intrinsic mefloquine resistance in regions of Africa where the drug had not been used (Oduola et al., 1992; White, 1994), although it now appears that this may have been due to pre-existing levels of quinine resistance. Resistance to mefloquine in the field was first noted in an area of Thailand (Boudreau et al., 1982) where quinine resistance was already widespread. The ease with which mefloquine resistance develops is exemplified by experiments, showing that mefloquine resistance readily can be induced by applying drug pressure during continuous passage of *P. berghei* through *Anopheles gambiae* (Fonseca et al., 1995).

Mefloquine has been used in higher doses as a solo treatment in much of Southeast Asia, although high levels of resistance to this therapy are now occurring there as well (ter Kuile et al., 1992). Mefloquine is still effective in most African countries and can be used in areas of chloroquine resistance. Mefloquine and halofantrine show a high degree of *in vitro* cross-resistance (Basco et al., 1995; Gay et al., 1990; Pradines et al., 1998; Rojas-Rivero et al., 1992; Basco and Le Bras, 1993), although evidence of *in vivo* cross resistance is limited. It indicates that increasing levels of resistance to mefloquine may limit the effective chemotherapy lifetime of both mefloquine and halofantrine.

ANTIFOLATES

Some of the most widely used antimalarial drugs belong to the folate antagonist class, albeit their role in malaria control is hampered by rapid emergence of resistance under drug pressure (Plowe et al., 1998). Inhibition of enzymes of the folate pathway results in decreased pyrimidine synthesis, hence, reduced DNA, serine, and methionine formation. Activity is exerted at all growing stages of the asexual erythrocytic cycle and on young gametocytes. Traditionally, antifolates are classified into two:

1. Type-1 antifolates (sulfonamides and sulfones) mimic p-aminobenzoic acid (PABA). They prevent the formation of dihydropteroate from hydroxymethyldihydropterin catalyzed by dihydropteroate synthase (DHPS) by competing for the active site of DHPS (a bifunctional enzyme in plasmodia coupled with 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine pyrophosphokinase [PPPK]).
2. Type-2 antifolates (pyrimethamine, biguanides and triazine metabolites, quinazolines) inhibit dihydrofolate reductase (DHFR, also a bifunctional enzyme in plasmodia coupled with thymidylate synthase [TS]), thus preventing the NADPH-dependent reduction of H2folate (DHF) to H4folate (THF) by DHFR.

Mode of action

The antifolate drugs inhibit either dihydrofolate reductase

(DHFR) (pyrimethamine, cycloguanil) or dihydropteroate synthase (DHPS) (sulfadoxine). These are two key enzymes in *de novo* folate biosynthesis; inhibition of this metabolic pathway leads to the inhibition of the biosynthesis of pyrimidines, purines, and some amino acids. Antifolate antimalarial drugs interfere with folate metabolism, a pathway essential to malaria parasite survival.

Mechanism of resistance

This class of drugs includes effective causal prophylactic and therapeutic agents, some of which act synergistically when used in combination. Unfortunately, the antifolates have proven susceptible to resistance in the malaria parasites. Resistance is caused by point mutations in DHFR and DHPS, the two key enzymes in the folate biosynthetic pathway which are targeted by antifolates. Resistance to these drugs arises relatively rapidly in response to drug pressure and is now common worldwide. Resistance to DHFR and DHPS inhibitors is conferred by single mutations of the gene encoding for the respective enzyme, resulting in substitutions in the amino acid chain. There are areas of the DHFR and DHPS genes with identified mutations that are found in isolates that fail to respond to pyrimethamine/sulfa treatment. These occur principally at codons 108, 51, 59, 164, and also occasionally 50, 140, and the ``Bolivian repeat'' of the DHFR gene and codons 436, 437, 540, 581, and 613 of the DHPS gene (Plowe et al., 1998). There is a broad correlation between increased frequency of such mutations and resistance to pyrimethamine/sulfa drugs across the world (Wang et al., 1997).

Status of resistance

For all the antifolate drugs, a high number of mutations may be an indicator of clinical resistance, but as the number of mutations becomes smaller, the accuracy in predicting clinical resistance decreases. Resistance to the fansidar combination is widespread, especially in Southeast Asia.

Fansidar has been used as a second-line drug in areas of chloroquine resistance; the loss of fansidar effectiveness caused by increasing resistance has important implications for Africa, where the number of inexpensive alternatives is limited. The development of resistance to sulfadoxine-pyrimethamine by *Plasmodium* parasites is a major problem for the effective treatment of malaria, especially *P. falciparum.* Although the molecular basis for parasite resistance is known, the factors providing the development and transmission of these resistant parasites are less clear. In Tanzania, increasing rate of chloroquine resistance led to change in its first line treatment of uncomplicated malaria to sulfadoxine-pyrimethamine. This antifolate combination seemed to be an effective and reasonable alternative but resistance to sulfadoxine-pyrimethamine was rapidly gaining ground, facilitated by the slow elimination from the body. High level (45%) of sulfadoxine-pyrimethamine treatment failures were recorded in Muheza, North East Tanzania by Mutabingwa et al. (2001). Nazila et al. (2000) observed that when a combination of pyrimethamine and sulfadoxine was used in Kenya, drug resistant parasites were selected rapidly. A study of pyrimethamine-sulfadoxine effectiveness was carried out between 1997 and 1999 at Kilify on the Kenyan Coast, and it concluded that prevalence of triple mutant DHFR-double mutant DHPS combination may be an operationally useful marker for predicting the effectiveness of pyrimethamine-sulfadoxine as a new malaria treatment.

Gatton et al. (2004) worked on evolution of resistance to pyrimethamine-sulfadoxine in *P. falciparum*. Their findings indicate three stages in the development of drug resistance.

The first is the collection of existing parasites with genetic mutations in the DHFR and DHPS gene and it was driven by long half-life of pyrimethamine-sulfadoxine combinations. The second stage involves the selection of parasites with allelic types of higher resistance within the host during an infection. And in the third stage, clinical treatment failure becomes prevalent as the parasites develop sufficient resistance mutation to survive therapeutic doses of the drug combinations. They emphasized on importance of correct treatment of confirmed malaria cases to avoid development of parasite resistance to pyrimethamine-sulfadoxine.

ARTEMISININ DERIVATIVES

For nearly 2,000 years, a cold-water extract of sweet wormwood (*Artemisia annua,* ‘‘qinghao’’) has been used in China for the treatment of fevers. The active ingredient of this plant was isolated in 1970 by Chinese scientists. Artemisinin (or qinghaosu) and its derivatives (artesunate, artemether, and arteether) have been used extensively in China and Southeast Asia, where there are high levels of resistance to the majority of the quinoline-containing drugs and to all the antifolate drugs (Meshnick et al., 1996). The artemisinin-type compounds in current use are either the natural extract artemisinin itself or the semi-synthetic derivatives (dihydroartemisinin, artesunate, artemether). They achieve higher reduction rates of parasitaemia per cycle than any other drug known to date (White, 1997).

Mode of action

Artemisinin and its derivatives are sequiterpene lactones.

Once administered, the artemisinin derivatives are hydrolyzed rapidly to the biologically active metabolite dihydroartemisinin. The mode of action of the artemisinin drugs has not been completely elucidated. The present knowledge is reviewed by Meshnick et al. (1996) and Cumming et al. (1997). The structure of artemisinin is unusual, and its activity is thought to depend on the presence of the endoperoxide bond, as molecules without it have no antimalarial activity (Brossi et al., 1988). The endoperoxide bond may interact with iron or heme, decomposing into free radicals (Meshnick et al., 1993, 1996; Paitayatat et al., 1997). Unlike many redox reactions, this process is not reversible, so a single drug molecule will produce only one free radical. The effect of free radicals on the malaria parasite is still not fully understood. Because the concentration of free radicals is insufficient to cause general membrane damage, one theory is that a ‘‘specific free radical target’’ exists (Meshnick, 1994). The artemisinin free radical can form a covalent bond with either heme or other parasite proteins (Yang et al., 1993, 1994) and an initial hypothesis was that a heme-artemisinin compound might inhibit the production of hemozoin. No evidence, however, of reduced quantities of hemozoin in artemisinin-treated *P. falciparum* cultures has been found (Asawamahasakda et al., 1994). Artemisinin also has been shown to bind to 6 specific *P. falciparum* proteins, one of which is a member of the translationally controlled tumor protein family but the precise effect of this protein alkylation on the parasite is still to be determined.

Mechanism and status of resistance

Artemisinin-resistant strains have been developed both in *P. falciparum* cultures (Inselburg, 1985) and in *P. yoelii* mouse models (Chawira et al., 1986). There also have been some indications of increasing *in vitro* resistance in field isolates (Gay et al., 1994). *P. falciparum* resistance to artemisinins, which was confirmed on the Cambodia-Thailand border in 2009, is now suspected in parts of Myanmar and Viet Nam. However, Artemisinin-based combination therapies (ACTs) remain highly effective in almost all settings, so long as the partner drug in the combination is locally effective. Artemisinin derivatives have a gametocytocidal activity (Peters, 1993), a feature that, in combination with their pharmacokinetic and pharmacodynamic properties, may well delay the development of drug resistance in the field. Resistance is difficult to induce experimentally, and is labile (low levels of resistance are achieved after sustained drug pressure) but not retained once drug pressure is removed (Peters and Robinson, 1999). Available data suggest that resistance to this class of compounds would be multigenic and share similarities with the quinoline family, as demonstrated *in vitro* on a series of parasite isolates (Meshnick, 1999).

ATOVAQUONE

The antimalarial activities of hydroxynaphthoquinones were discovered during World War II. Atovaquone is the first effective compound in this class. Currently, it is being marketed as Malarone, which contains a fixed combination of atovaquone and proguanil.

Mode of action

Atovaquone {2-[trans-4-(40-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone}, hydroxynaphthoquinone is used for both the treatment and prevention of malaria in a fixed combination with proguanil. Whilst known to act primarily on mitochondrial functions, its mode of action and synergy with proguanil are not completely understood. This matter is further complicated by the diverse functions of mitochondria in various organisms and by technical difficulties with experiments. It is generally agreed that atovaquone acts on the mitochondrial electron transfer chain, although more recently, its activity and synergy with proguanil has been ascribed to its interference with mitochondrial membrane potential. Atovaquone inhibits cytochrome *c* reductase activity in *P. falciparum* (Fry and Pudney, 1992). Atovaquone is a ubiquinone analogue that binds to the cytochrome *bc1* complex of the parasite mitochondrial electron transport chain. The malaria mitochondria electron transport chain disposes of electrons generated by dihydroororate dehydrogenase during the synthesis of pyrimidines (Gutteridge et al., 1979) and the inhibition of this process by atovaquone may kill the parasite (Hammond et al., 1985). More recently, it has been shown in *P. yoelli* that atovaquone also dissipates the mitochondrial membrane potential of the parasite which may kill the parasite by initiating a process similar to apoptosis.

Mechanism and status of resistance

When atovaquone was first used in clinical trials in Thailand, the treatment failure rate was 33%, regardless of duration of therapy (Looareesuwan et al., 1999). This high level of treatment failure suggests that either a natural background of resistant mutants exists or resistance arises rapidly by the acquisition of point mutations in the cytochrome *b* gene. Mutations in cytochrome *b* have been found in atovaquone-resistant *Pneumocystis carini* and *P. yoelii* strains, indicating that they may be the cause of atovaquone resistance (Walker et al., 1998; Srivastava et al., 1999). Because of the high rate of treatment failure, atovaquone has been combined with other drugs, including proguanil, doxycycline, and tetracycline. All of these combinations yielded high cure rates (Looareesuwan et al., 1999; Redloff et al., 1996), and the atovaquone-proguanil combination (Malarone)

also is effective as a prophylactic.

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Full Length Research Paper

Activation of caspase-activated deoxyribonuclease and neuroprotective effect of caspase-3 inhibitor after focal cerebral ischemia-reperfusion injury

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Caspase-3 is a key enzyme to execute apoptosis and may be cause internucleosomal DNA fragmentation in ischemic neurons. However, whether caspase-3 inhibitor can directly inhibit caspase-3–dependent deoxyribonuclease activity and prevent neuronal apoptosis following cerebral ischemic is unknown. In this study, we detected the caspase-3 and CAD protein, as well as the frequencies of neuronal apoptosis in both model control group (DMSO injection) and treatment group (Ac-DEVD-CHO injection) after focal cerebral ischemia-reperfusion in rats. Our data showed that caspase-3 and CAD protein were detectable, and apoptotic-like neuronal death occurred following cerebral ischemic in both groups. However, these results obtained were inhibited by Ac-DEVD-CHO in treatment group as compared with model control group. Taken together, these data further support that the pathway of caspase-3–dependent CAD activity and neuronal apoptosis is an important mechanism in ischemic neuronal injury, and Ac-DEVD-CHO has the neuroprotective effect to a certain degree.

Key words: Caspase-3, caspase-activated deoxyribonuclease, Ac-DEVD-CHO, apoptosis, cerebral ischemia.

INTRODUCTION

Neuronal injury can be caused by many mechanisms after cerebral ischemia, in which neuronal apoptosis mediated by caspases family is an important one (Martin and Green, 1995; Alnemri, 1997; Salvesen and Dixit, 1997; Nunez et al., 1998; Porter and Janicke, 1999; Le et al., 2002). The caspase family includes fourteen members, and caspase-3 plays a key role in the apoptotic pathway among them (Hara et al., 1997; Yakovlev et al., 1997; Chen et al., 1998; Wu et al., 2011; Li et al., 2012). Caspase-activated deoxyribonuclease (CAD), which is also named DNA fragmentation factor (DFF40), participates in the apoptotic cascades as an important effector of caspase-3 (Liu et al., 1997; Inohara et al., 1999; Cao et al., 2001). In normal cells, CAD and its endogenous inhibitor of caspase-activated deoxyribonuclease (ICAD) are conjugated into an inactive dipolymer. During apoptosis, ICAD is cleaved by caspase-3 and released from the ICAD/CAD complex, leading to activation of CAD and subsequent DNA fragmentation (Cory, 1998; Sakahira et al., 1998).

Previous studies have suggested that caspase-3 dependent CAD activity play an essential role during neuronal apoptosis after ischemia in the brain (Chen et al., 1998; Tsukada et al., 2001; Luo et al., 2002; Abas et al., 2010; Yan et al., 2010). Hence, antiapoptotic mechanisms through caspase inhibition may play neuroprotective role in the brain after focal ischemic injury.

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Le et al. (2002) has reported that caspase-3 gene knockout mice were more resistant to ischemic injury than Wistar mice; however, the study using caspase-3 gene knockout mice was limited by the lack of practicability. Therefore, in this study we aimed to demonstrate the neuroprotective effect of caspase-3 inhibitor that will be more likely utilized in future, in a rat model of focal cerebral ischemia and reperfusion.

MATERIALS AND METHODS

Animal grouping

Sixty-four adult male Wistar rats, weighing 200 - 250 g were randomly divided into the model control group (n = 32) and treatment group (n = 32). They were observed at 6, 12, 24, 48 and 72 h of ischemia, respectively. One more rat was included in the 48 and 72 h groups for electron microscopic observation. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal care and experimental protocols were approved by the animal ethics committee of 2nd Affiliated Hospital of Harbin Medical University.

Model preparation

Focal ischemia was induced by occlusion to the right middle cerebral artery (MCAO) as described previously (Nagasawa and Kogure., 1989). After the anesthesia with 10% chloral hydrate (300 mg/kg) in rats, a nylon thread (0.17 mm in diameter and 0.24 mm in distal cylinder) was inserted into the lumen of the internal carotid artery and advanced to the origin of the MCAO. Then, rats were subjected to intra-cerebral ventricular infusion. The model control group was injected with 4 µL of 0.3% dimethyl sulfoxide (DMSO) for controls, whereas treatment group was injected with 5 µg of Ac-DEVD-CHO (Promega, USA) containing 4 µL 0.3% DMSO. The thread was removed 1 h later to allow for reperfusion. The rats were enrolled according to neurological findings, including left forelimb flexion or circling 1 h after MCAO.

Sample preparation

One rat was sacrificed at 48 and 72 h in both groups. The temporo-parietal lobe cortex tissue of about 1 mm was taken from the right cerebral hemisphere. It was then fixed with glutaraldehyde for electron microscopic observation (n = 4). Other rats were decapitated under anesthesia at different time points (n = 60). After 12 h external fixation, coronary brain slices (2 mm anterior and posterior to the optic chiasma) were cut and embedded with paraffin for section preparation.

Hematoxylin and Eosin (HE) and immunohistochemistry staining

Tissue sections were performed with routine HE staining. Immunohistochemistry was performed according to the instructions indicated in the kit (PV-6001, Zhongshan, China). Cells with a buffy-stained nucleus or cytoplasma were defined positive. Tissue sections were deparaffinized, hydrated and then incubated with 3% H_2O_2 deionized water for 5 - 10 min. They were incubated with at 37°C rabbit anti-mouse caspase-3 and CAD polyclonal antibodies for 1 - 2 h. After the incubation with horseradish peroxidase (HRP)-conjugated mouse anti-rabbit IgG antibody at room temperature (37°C) for 20 - 30 min, the sections were DAB-stained. The number of caspase-3 and CAD positive cells was recorded in 5 randomly chosen areas on the temporo-parietal cortex tissue under the microscopy (40X objective), and then mean values were calculated.

TUNLE staining

The staining procedures were performed according to the instructions indicated in the kit (Roche, USA). Sections adjacent to those for immunohistochemistry were collected, processed with hydrogen peroxide-methanol and citrate sodium, and then incubated with TUNLE reaction mixture at 37°C for 60 min. They were incubated with peroxidase-labeled anti-fluorescence antibodies at 37°C for 30 min. The substrates were DAB-stained at room temperature away from light. The shade of staining was controlled under a microscope. The sections were dehydrated, cleared, and then mounted regularly. Neurons with buffy-stained granules in the nucleus were defined positive. The number of apoptotic neurons was recorded in 5 randomly chosen areas on the temporo-parietal cortex tissue under the microscopy (40x objective), and then mean values were calculated.

Electron microscopy

The processed sections were observed under a JEM-1220 transmission electron microscope, and photographs were taken.

Statistical analysis

All data were presented as mean ± SD. Comparison between paired and unpaired groups was based on Bartlett's test for homogeneity of variances, and analysis of variance (ANOVA) or nonparametric ANOVA test was chosen by SPSS 13.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Pathologic features after ischemia

Using HE staining, we observed no obvious morphological change at 6 h after reperfusion in the model control group (Figure 1). At 12 h, karyopyknosis was found in a few nerve cells and moderate edema was found around the neurons. With the prolongation of time, the cytoplasm acidophily increased gradually, and numerous apoptotic nerve cells were observed at 48 h (Figure 1). In the treatment group, cellular edema was relieved at the early stage compared to the model control group; however, no obvious difference in apoptosis was found at the late stage.

By electron microscopy, intra-neuronal chromatolysis and formation of vacuole-like structure was found in both groups at 24 and 48 h (Figure 2). The structure of the organelles in the cytoplasm changed, and lipofuscin granules, blurred mitochondrial crista and decreased metrical density could be seen. The vascular nerves

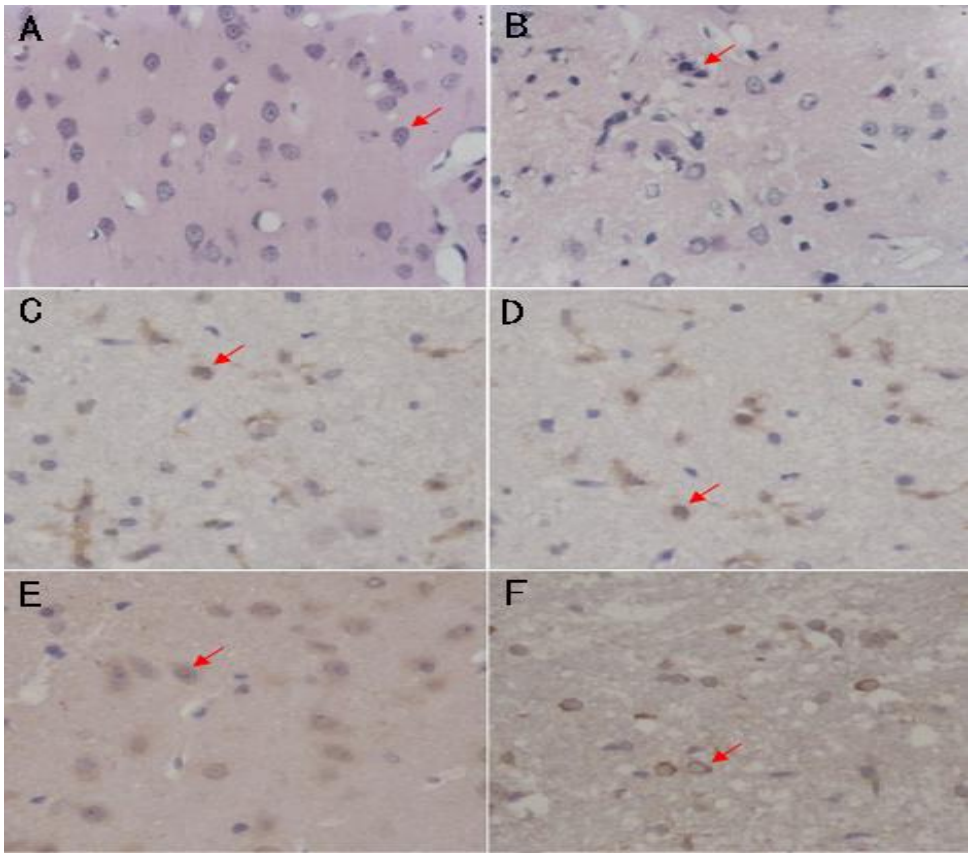

Figure 1. Representative photomicrographs (400× magnification) of HE staining (A and B) and immuno-histochemistry staining for caspase-3 (C and D) and CAD (E and F). HE staining: (A) Normal nerve cells (red arrows), (B) numerous apoptotic nerve cells (red arrows) after 48 h reperfusion. Caspase-3 staining: (C and D) positive cells were stained brown (red arrows), both in the cytoplasm and nucleus, higher caspase-3 positive cells in model control group (C) than in treatment group (D) after 48 h reperfusion. CAD staining: (E and F) positive cells were stained brown (red arrows), CAD protein stained concentrated around the nucleus in the treatment group (F) or spread all over the nucleus in the model control group (E) after 48 h reperfusion, and comparison of the treatment group with model control group showed that CAD positive cells decreased significantly.

showed obviously broadened interspaces, the fibers were lysed, and the lumens were narrowed. Some cell membranes were ruptured, and necrosis and apoptosis were present. However, no typical apoptotic bodies were found.

Caspase-3 protein alteration after ischemia

In the model control group, a few caspase-3 positive cells were observed at 6 h after reperfusion, and they were significantly increased from 12 to 72 h, and reached the peak at 24 h. Caspase-3 protein was mainly located in cytoplasm at 12 h, and was shown in cytoplasm and nucleus at 24, then transferred from cytoplasm into the nucleus at 48 h. In the treatment group, the changes showed a similar trend to that in the model control group, but caspase-3 positive cells were significantly decreased 12 to 48 h ($P < 0.05$) (Figure 1). The data were shown in Figure 3 and Table 1.

CAD protein alteration after ischemia

In the model control group, CAD positive cells were rarely detected at 6 h. At 12 h, CAD protein began to concentrate around the nucleus in a few cells. At 48 h, it spread all over the nucleus, and the frequency of CAD positive cells reached the peak (Figure 1). In the treatment group, CAD positive cells also reached the peak at 48 h, but significant decrease were found from 24 to 72 h ($P < 0.05$) and not at 6 h and 12 h ($P > 0.05$)

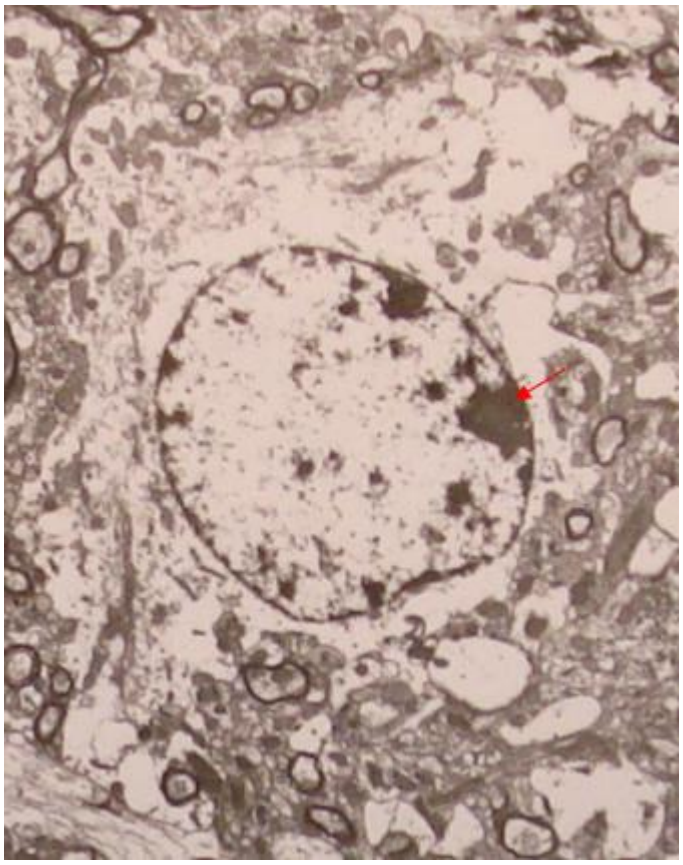

Figure 2. Electron micrographs showing the formation of vacuole-like structure and chromatin condensed in margin (red arrows) after 48 h reperfusion.

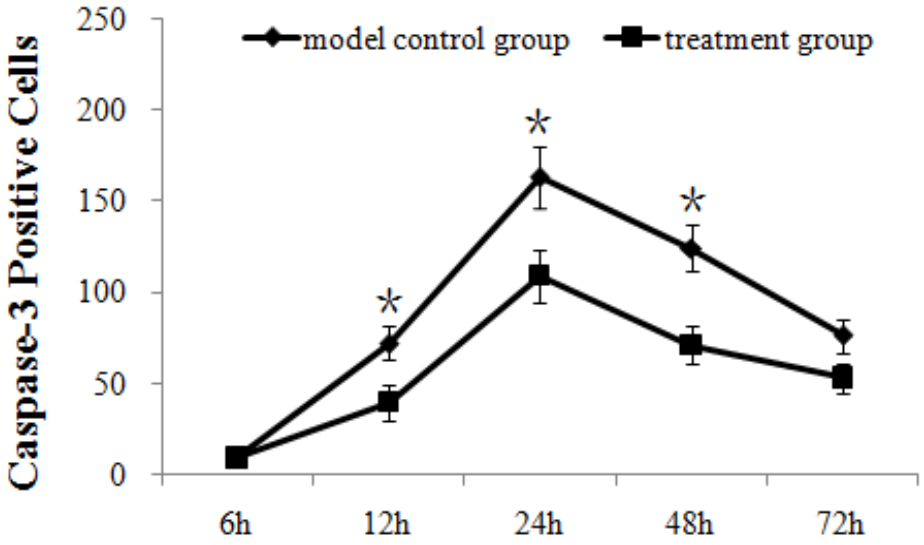

Figure 3. Comparisons of the caspase-3 positive cells between the model control and treatment group at each time point after reperfusion. *P < 0.05 compared with model control group at the same time point.

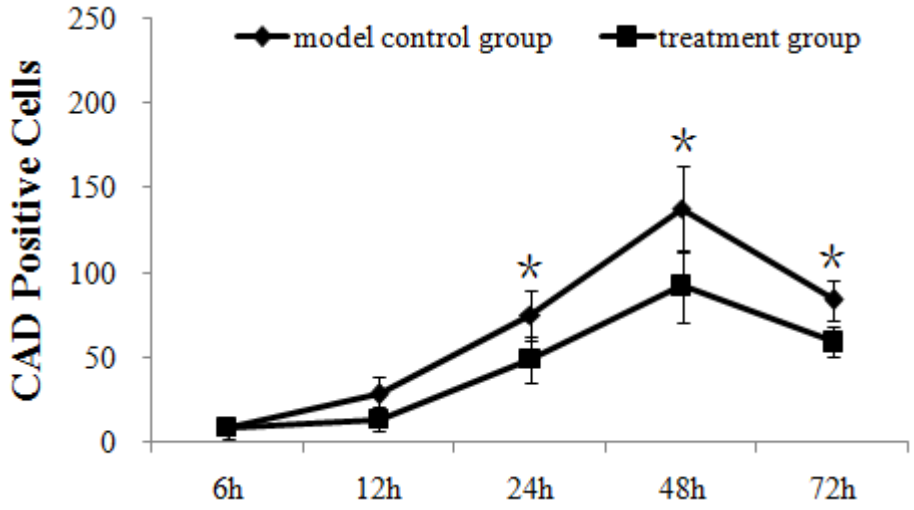

Figure 4. Comparisons of the CAD positive cells between the model control and treatment group at each time point after reperfusion. *P < 0.05 compared with model control group at the same time point.

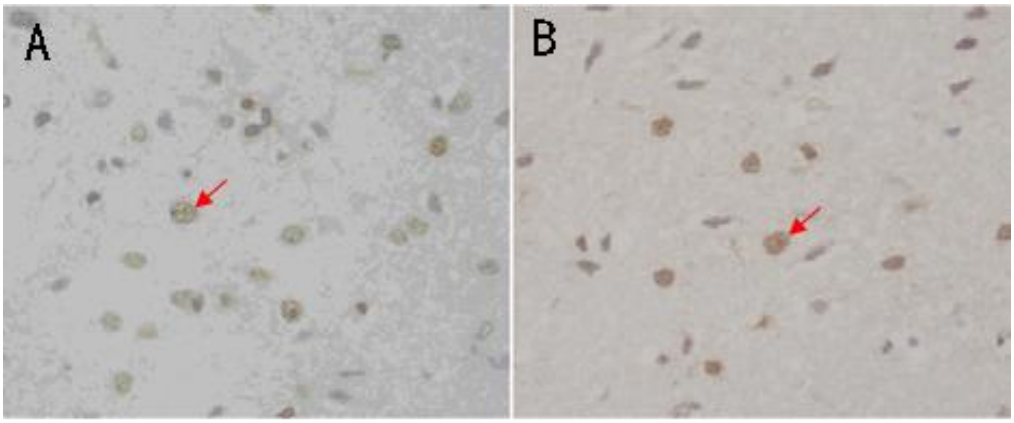

Figure 5. Representative photomicrographs (400× magnification) of TUNEL staining from model control group (A) and treatment group (B) after 72 h reperfusion. TUNEL positive cells showed a condensed, shrunken, or fragmented nucleus (red arrows). TUNEL positive cells were significantly reduced in treatment group as compared with model control group.

(Figure 1). The data are shown in Figure 4.

Neuroprotective effect of Ac-DEVD-CHO

In situ apoptosis was detected using TUNLE staining. In the model control group, few positive cells were found at 6 h. With the prolongation of time, the number of apoptotic cells increased, and reached the peak at 72 h. In the treatment group, the number of apoptotic cells at 6 h and 12 h showed no significant difference (P > 0.05), while at 24 to 72 h, the results displayed significantly decreased as compared with model control group (P < 0.05), which was especially obvious at 72 h. The alteration suggests that Ac-DEVD-CHO inhibit the induction of apoptosis after an ischemic insult. Representative TUNLE staining is presented in Figure 5, and the data are shown in Figure 6 and Table 1.

DISCUSSION

Utilizing a rat model of MCAO, the neuroprotective effects of caspase-3 inhibitor Ac-DEVD-CHO was confirmed in our study. The major results demonstrated that Ac-DEVD-CHO injection prior to reperfusion reduced up-regulation

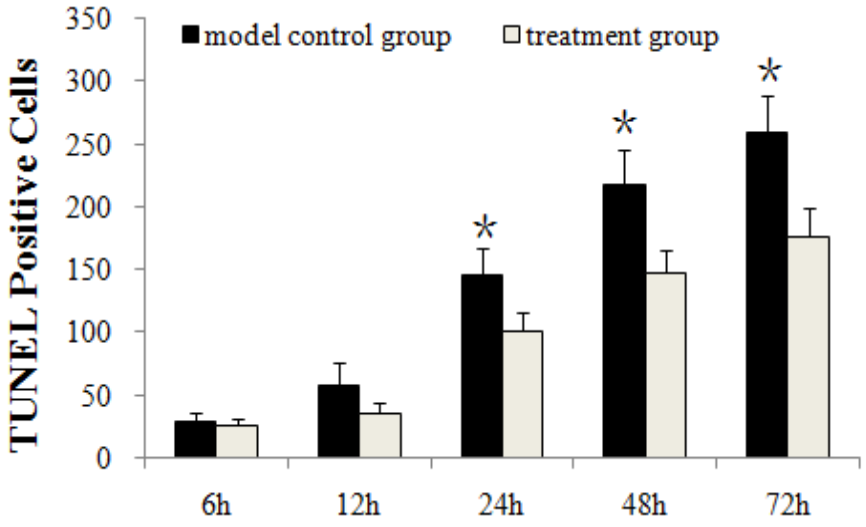

Figure 6. Comparisons of the TUNLE positive cells between the model control and treatment group at each time point after reperfusion. *P < 0.05 compared with model control group at the same time point.

Table 1. Caspase-3, CAD and TUNEL positive cells in the model group and treatment group at each time point after reperfusion.

Positive cells	Time after reperfusion (h)	Model control group	Treatment group	P value
Caspase-3	6	10.0 ± 3.4	9.2 ± 3.2	> 0.05
	12	72.0 ± 9.1	39.3 ± 10.0	< 0.05
	24	163.4 ± 16.7	109.1 ± 14.5	< 0.05
	48	124.3 ± 12.7	70.8 ± 10.3	< 0.05
	72	76.3 ± 9.4	52.9 ± 8.3	> 0.05
CAD	6	8.4 ± 6.0	8.5 ± 4.4	> 0.05
	12	28.3 ± 9.9	13.6 ± 6.8	> 0.05
	24	74.7 ± 14.9	49.2 ± 13.5	< 0.05
	48	137.9 ± 25.5	92.2 ± 21.0	< 0.05
	72	83.7 ± 12.1	59.1 ± 8.9	< 0.05
TUNEL	6	28.9 ± 6.6	26.4 ± 4.7	> 0.05
	12	58.4 ± 18.0	35.7 ± 8.8	> 0.05
	24	145.5 ± 22.3	101.1 ± 15.3	< 0.05
	48	217.2 ± 27.7	147.2 ± 18.2	< 0.05
	72	258.9 ± 29.7	176.1 ± 22.3	< 0.05

*P < 0.05 compared with model control group at the same time point.

of caspase-3 and CAD and decreased neuronal apoptosis following cerebral ischemia. Accumulating evidence indicate that the caspase-family is the promoter and implementer of apoptosis in mammalian cells, and caspase-3 is the most critical downstream apoptosis protease in the caspase cascade "waterfall" (Le et al., 2002; Cho and Toledo-Pereyra, 2008). In the present study, we found that there were significant increases in caspase-3 and CAD protein and apoptotic cells in a time-dependent manner after focal ischemia and reperfusion in the brain. The up-regulated expression of caspase-3 protein first reached the peak (at 24 h, and then began to

decrease), then CAD protein spread all over the nucleus and reach the peak and at 48 h. However, a large quantity of TUNEL positive cells appeared up to 72 h after ischemia-reperfusion. These results revealed a dynamic apoptotic process encompassing caspase-3 activation, caspase-3 nuclear transfer, CAD protein up-regulation, CAD translocation, DNA degradation (TUNLE positive cells). This process is an important pathway to neuronal injuries in rats after cerebral ischemia-reperfusion. The time-dependent relationship between caspase-3 and apoptosis further supports the theory that caspase-3-mediated mechanism play a key role in the final execution of neuronal apoptosis in various forms of central nervous system injuries (Yakovlev et al., 1997; Namura et al., 1998; Springer et al., 1999; Zhang et al., 1999; Citron et al., 2000; Clark et al., 2000; Sharp et al., 2000; Cao et al., 2001; Graham and Chen, 2001; Tsukada et al., 2001).

Furthermore, we observed that caspase-3 inhibitor Ac-DEVD-CHO diminished CAD activation and prevented endogenous DNA fragmentation after ischemia. In brief, 4.5 µg of z-DEVD-fmk (also a caspase-3 inhibitor) has the best inhibitory effect on caspse-3 activity and the optimal time window is within 6 h after ischemia in a 30 min focal ischemic model (Luo et al., 2002; Wei et al., 2004). Based on the aforementioned views, in the current study, 5 µg of Ac-DEVD-CHO was selected as the therapy dose. Despite inhibition of the caspase-3 activity by Ac-DEVD-CHO, we observed a large number of apoptotic cells in the treatment group after ischemia. Therefore, it is certain that neuronal apoptosis is a complex process in which many factors are involved, and the inhibition of CAD can only partially inhibit the process (Didenko et al., 2002; Nielsen et al., 2008). Apoptotic response originates from the release of cytochrome oxidase C, and then proceeds with the activation of caspase-3 and other multi-target genes (such as PARP, CAD and other nucleate endonucleases), while CAD is only one of the multiple target genes. This is consistent to the findings of Le et al. (2002) that genetic deletion of caspase-3 renders neurons more resistant to ischemic stress, but features of apoptotic-like neuronal death remained be observed both in vivo and in vitro, despite deletion of the caspase-3 gene. These evidences suggest that caspase-independent mechanisms could play a critical role in the death process as well.

In summary, caspase-3 dependent CAD activity and neuronal apoptosis is induced in the brain after focal ischemia and reperfusion. Caspase-3 inhibition can suppress ischemic injury and play the role of neuroprotection. Taken together, blockage of caspase may be an effective treatment for stroke in future.

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Full Length Research Paper

Antitumor effects and mechanisms of total saponin and total flavonoid extracts from *Patrinia villosa* (Thunb.) Juss

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***Patrinia villosa* (Thunb.) Juss is a Chinese edible herbal widely used in China for treatment of carbuncles, acute appendicitis, hepatitis and stasis for hundreds of years. In this study, the antitumor effects and the possible mechanisms of total saponin extract from *P. villosa* (SPV) and total flavonoid extract from *P. villosa* (FPV) were investigated in four cancer cell lines including mouse melanoma cell line B16, MCF-7 human breast cancer cells, Hela human epithelial cervical cancer cells and L1210 mouse lymphocytic leukemia cells. The antiproliferative effects of SPV and FPV on these cells were observed by 3-(4,5-Dimethyithiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The cell cycle was detected by flow cytometry. The expression of CDK4 and cyclin D1 were measured by western blot. The results of MTT assay suggested that FPV showed much stronger antiproliferative effects on L1210 cells in a dose-dependent manner. On the other hand, SPV showed better antiproliferative effect than FPV on the other three cell lines in a dose-dependent manner. The mechanism of antitumor effect of SPV and FPV might be the inhibition of expression of CDK4 and cyclin D1, and accordingly arrested four cancer cell lines in G0/G1 phase, decreased the number of cells in S phase, and finally induced antiproliferative effect. In summary, pharmacological data obtained from this study suggested that SPV and FPV possessed cancer chemopreventive potential on different types of cancer cells. These results were much more favorable on bioactivity-guided isolations of SPV and FPV.**

Key words: *Patrinia villosa* (Thunb.) Juss, saponins, flavonoids, antitumor, mechanism.

INTRODUCTION

Patrinia villosa (Thunb.) Juss (Valerianaceae), an herbaceous plant, is distributed mainly in East Asia and Northeast North America. It is a commonly used herbal medicine in China for treatment of carbuncles, acute appendicitis, hepatitis, amygdalitis, angina parotidea, anthracia, stasis, intestinal abscess, postpartum pain, dysmenorrhoea and endometriosis for hundreds of years. Little biological activity of *P. villosa* constituents was evaluated, although *P. villosa* was reported to be a rich source of flavonoids (Peng et al., 2006a, 2005a, 2006c, d, 2005c). The proangiogenic activity of the extract of *P. villosa* is confirmed by an *in vitro* mouse aortic ring assay and an *in vivo* murine hindlimb ischemia model (Jeon et al., 2010). Yang et al. (2006) showed that the whole extracts of *P. villosa* extract improved myocardial and cerebral oxygen consumption. Moreover, saponin extract from *P. villosa* effectively reduced the weight of U14 cervical tumor, inhibited proliferating cell nuclear antigen (PCNA) of tumor cell, decreased the expression of mutant p53 and bcl-2 protein (Zhang et al., 2008). However, little is known about a systematic comparison of antitumor effects on different cell lines between the total saponin extract from *P. villosa* (SPV) and the total flavonoid extract from *P. villosa* (FPV) and the signaling mechanisms responsible for their biological activity. Therefore, in this study, the antitumor effects and the possible mechanisms of SPV and FPV were investigated

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in four cancer cell lines including mouse melanoma cell line B16, MCF-7 human breast cancer cells, Hela human epithelial cervical cancer cells and L1210 mouse lymphocytic leukemia cells.

MATERIALS AND METHODS

Chemicals and reagents

Cell culture media, phosphate buffer solution (PBS) and fetal bovine serum (FBS) were from Gibco (Tulsa, OK, USA). All cell culture plastic ware (COSTAR®) was purchased from Corning (New York, USA). 3-(4,5-Dimethyithiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was from Promega Corporation (WI, USA). The Via Count Assay kit, the Guava Cell Cycle kit and enhanced chemiluminescence detection kit were from Millipore (Millipore Corporation, Hayward, CA). Antibodies for CDK4 (sc-200), cyclin D1 (sc-575) and secondary antibodies were obtained from Santa Cruz Biotechnology Inc. (CA, USA). Macroporous resin D101 was purchased from the Chemical Plant of NanKai University (Tianjin, China).

Plant

The whole plants of *P. villosa* were collected in September, 2009 at Changbai Mountains, Tonghua, People's Republic of China, and identified by Associate Professor Hong Zhao, Marine College, Shandong University at Weihai. A voucher specimen (No. CB 2009007) was deposited in the Herbarium of Laboratory of Botany, Research Center of Medical Chemistry and Chemical Biology, Chongqing Technology and Business University.

Extraction

Extraction of SPV

Dried *P. villosa* rhizomes were pulverized and extracted with 95% ethanol. The solvent of the extract solution was evaporated under vacuum. The dried extract was dissolved in water, and then extracted with petroleum ether. The water phase was absorbed by macroreticular resin D101. The SPV was eluted with 75% ethanol aqueous solution, followed by lyophilizing to give the extract (Xu et al., 2006).

Extraction of FPV

Dried *P. villosa* rhizomes were pulverized and extracted with 75% ethanol by Suoshi extraction method and the flavonoids content was measured using ultraviolet (UV) spectrum (Xu et al., 2004).

Cell lines

The mouse melanoma cell line B16, MCF-7 human breast cancer cells and Hela human epithelial cervical cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine and 10% foetal calf serum. L1210 mouse lymphocytic leukemia cells were cultured in RPMI 1640 media supplemented with 2 mM L-glutamine and 10% foetal calf serum. All cells were cultured in media supplemented with 1% penicillin/streptomycin and kept at 37°C and humidified in 5% CO_2.

Growth inhibition analysis

SPV and FPV were dissolved in dimethyl sulfoxide (DMSO). Cells were seeded in 6-well plates. After 1 day, various concentrations of extracts were added to the wells. The final concentration of DMSO was 0.5%. Controls were exposed to culture medium containing 0.5% DMSO without drugs. Cell proliferation was evaluated using an MTT assay. The dark blue formazan crystals formed in intact cells were solubilized with DMSO, and optical densities at 570 nm was measured with a TECAN infinite M200 (TECAN Group Ltd).

Cell proliferation analysis

Cells were routinely seeded into 6-well plates at 2×10^4 cells/well and incubated at 37°C for 24 h. Cells were incubated with various concentrations of extracts for 48 h. At the end of this time, the cells were trypsinized to produce a single cell suspension, and the viable cell number in each well was counted using the Via Count Assay (Guava Technologies, Hayward, CA).

Cell cycle analysis

Cells were trypsinized, centrifuged, and fixed in ice-cold 70% ethanol at 4°C for 16 to 24 h. Evaluation of cell cycle was carried out using the Guava Cell Cycle Kit. Cell pellets were resuspended in 200 µl Guava Cell Cycle Reagent for 30 min at room temperature, shielded from light. The stained cells were analyzed using the Guava EasyCyte™MINI (Guava Technologies, Hayward, CA) and data were analyzed using the Guava CytoSoft software package.

Western blot analysis

For Western blot analysis, 30 µg samples of cell extract were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted. The following primary antibodies were used: rabbit polyclonal antibodies to CDK4 (sc-200), cyclin D1 (sc-575) at 1:5000 dilutions for 2 h. The antigen-antibody complex was then detected by incubating the membrane for another 1 h in buffer containing a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit immunoglobin G (IgG) secondary antibody. Excess antibody was washed off with 20 mM TBST (20 mM Tris, 150 mM NaCl, pH 7.5, and 0.1% Tween 20). Detection was performed using enhanced chemiluminescence detection kit. Band intensities were quantified with the software of quantity one.

Statistical analysis

Results are given as means ± standard deviation (SD). Student's two-tailed *t*-test or one-way analysis of variance was used to determine significant differences between two means ($P<0.05$ or $P<0.01$).

RESULTS

Antiproliferative effects of SPV and FPV on different cancer cell lines

The cell growth inhibitory effects of SPV and FPV on four cancer cell lines were determined using the MTT assay. As shown in Figure 1A, compared with SPV, FPV showed much stronger antiproliferative effects on L1210

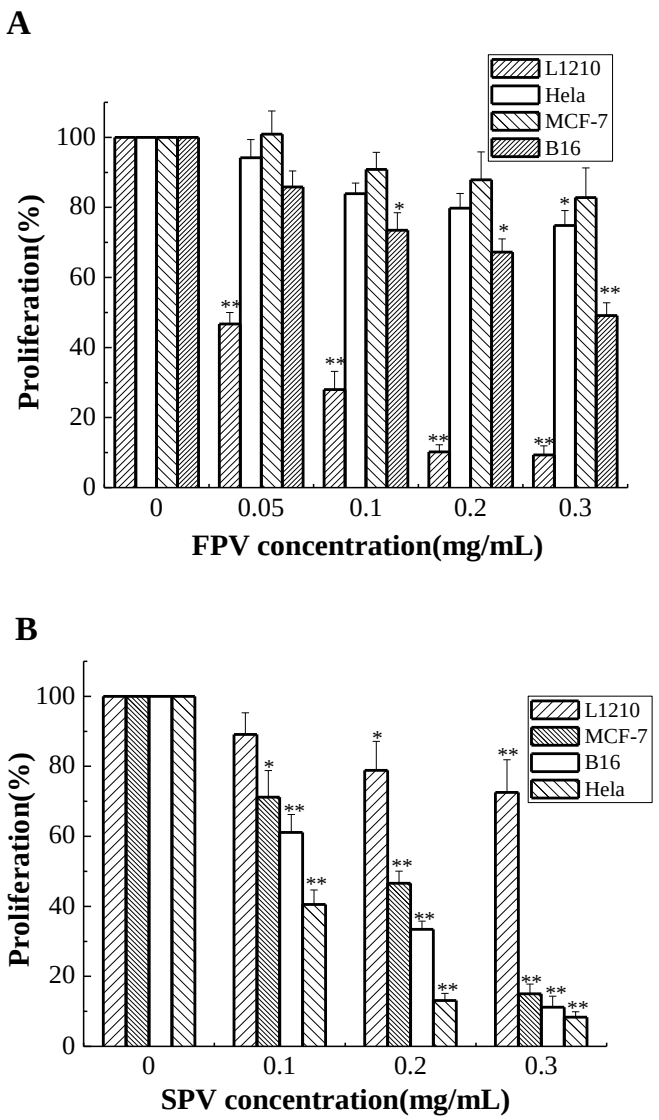

Figure 1. Effects of SPV (A) and FPV (B) on proliferation of different cancer cell lines assayed by MTT method. Cells were treated with indicated concentration extracts for 48 h. L1210 cells are more sensitive to FPV, while Hela, MCF-7 and B16 cells are more sensitive to SPV. *P<0.05 and **P<0.01 versus control.

cells in a dose-dependent manner (P<0.01). On the other hand, SPV showed better antiproliferative effects than FPV on the other three cell lines in a dose-dependent manner (P<0.01), including Hela, MCF-7 and B16 cells (Figure 1B). The most potent antiproliferative effects were observed on Hela cells treated with SPV and L1210 cell lines treated with FPV. At 0.1 mg/ml for 48 h, the FPV inhibited cell growth by 72.1% in the L1210 cell line, and SPV by 59.5% in the Hela cells, respectively. The results of flow cytometry analysis showed the same trend as with MTT assay. As shown in Figure 2, the percentage of dead cells increased significantly after 48 h of treatment, and the inhibition rates of the cell proliferation were similar with MTT assay results for both extracts.

Effects of SPV and FPV on cell cycle

To explore the potential mechanism of SPV and FPV inhibited cell growth, the cell cycle profile was assayed by flow cytometry after staining with propidium iodide (PI). Based on the MTT and ViaCount assay data, the effect of SPV on Hela, MCF-7, and B16 cells cycle and of FPV on L1210 cells cycle were evaluated.

As shown in Table 1, the S-phase population of four different cell lines significantly decreased in dose-dependent manners (P<0.01). Compared with the control, cell cycle profile was changed after 48 h treatment. FPV treatment obviously decreased S-phase cells of L1210 cells at 0.02 and 0.05 mg/ml. However, the 0.05 and 0.1 mg/ml of SPV decreased S-phase cells of other three cell lines.

Effects of SPV and FPV on expression of CDK4 and cyclin D1

It was reported that cyclin D1 and its catalytic partner CDK4 played important roles in the G1/S checkpoint of the cell cycle. To observe additional information involved in the cell cycle regulations, the expression of cyclin D1 in Hela, MCF-7, B16 and L1210 cancer cells was evaluated (Figure 3). FPV down-regulated the expressions of cyclin D1 and CDK4 in a dose-dependent manner in L1210 cells. In the same way, SPV down-regulated the expressions of the two proteins in a dose-dependent manner in Hela, MCF-7 and B16 cells. As shown in Figure 3B compared to the control, the incubation in Hela cells with 0.05 and 0.1 mg/ml SPV for 48 h, resulted in decreased of CDK4 to 39.6 and 19.5% and cyclin D1 also decreased to 13.2 and 8.1%, respectively. In L1210 cells, incubation with 0.03 and 0.05 mg/ml FPV for 48 h, resulted in decreased of CDK4 to 59.7 and 22.0% and cyclin D1 also decreased to 49.0 and 21.1%, respectively.

DISCUSSION

P. villosa is used to treat bowel cancer and carcinoma of the bladder. However, there are no report about a systematic comparison of antitumor effects on different cell lines between SPV and FPV, although many of the components have been separated (Peng et al., 2006a, b, 2005a, b) and antitumor effects of SPV on mice bearing U14 cervical cancer had been researched (Zhang et al., 2008).

In this study, we compared the antiproliferative potential of SPV and FPV using four cancer cell lines. The cancer cell lines used includes four types of cancers, mouse melanoma cell line B16, MCF-7 human breast cancer cells, Hela human epithelial cervical cancer cells and L1210 mouse lymphocytic leukemia cells. The results of MTT assay showed that the FPV inhibited the

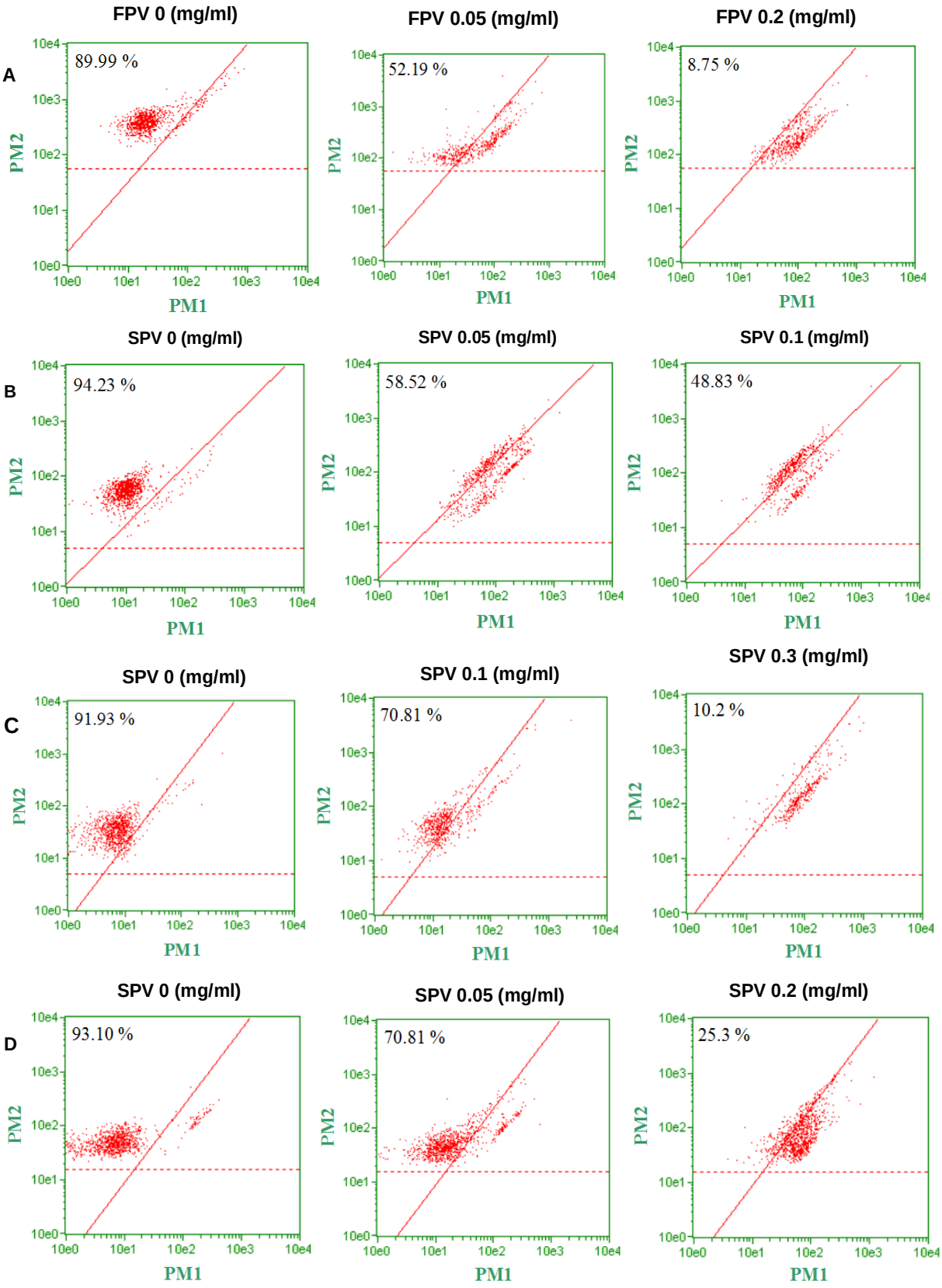

Figure 2. Effects of SPV and FPV on cell apoptosis of different cancer cell lines (L1210 (A), Hela (B), MCF-7 (C), B16 (D)) assayed by flow cytometry. After treated with different concentration SPV and FPV for 48 h, the results of flow cytometry analysis showed the same trend as with MTT assay.

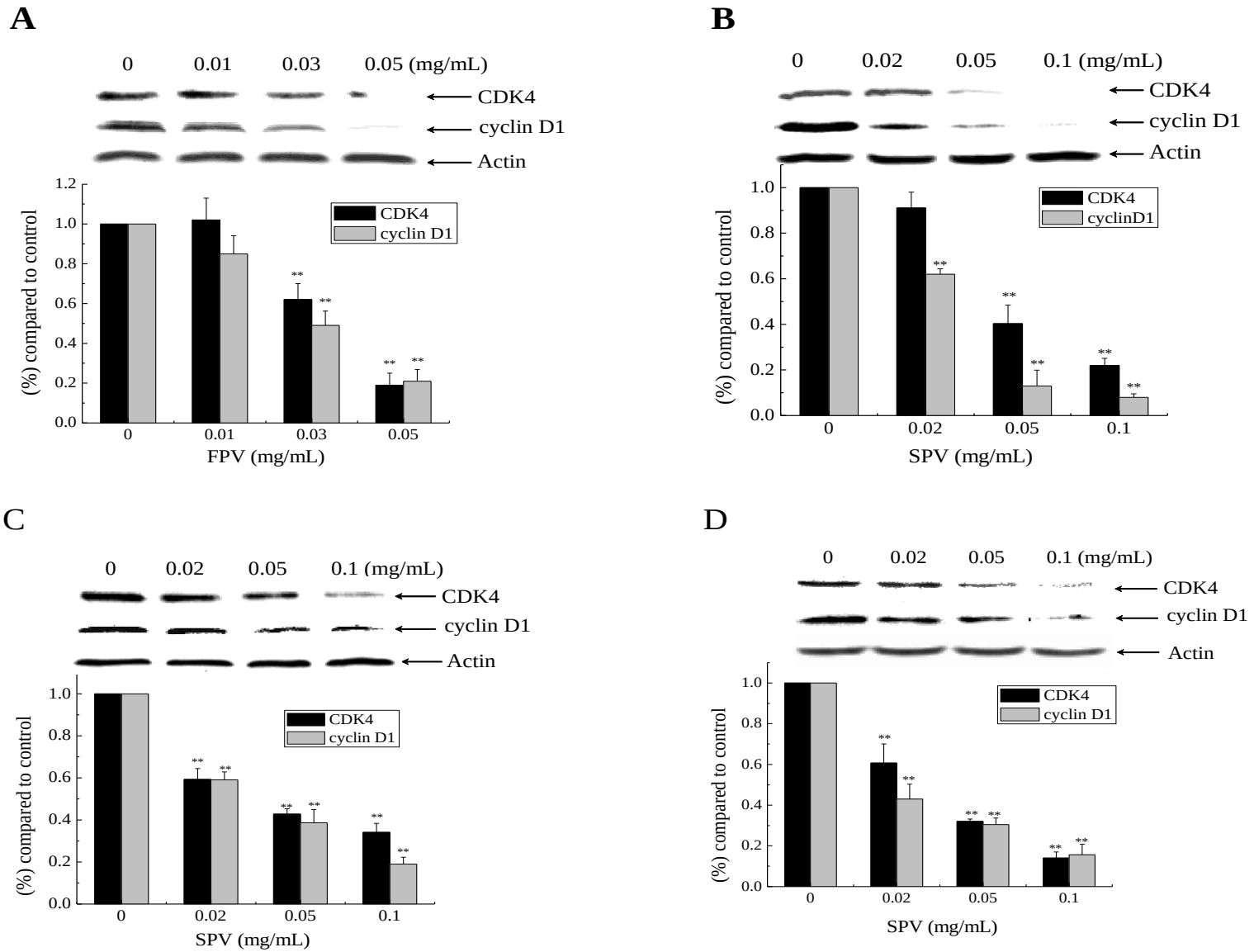

Figure 3. Effects of SPV and FPV on expression of cell cycle-related proteins CDK4 and cyclin D1 in four different cancer cell lines (L1210(A), Hela(B), MCF-7(C), B16(D)). After treated with different concentration SPV and FPV for 48 h, equal cell lysates were separated on 10% SDS-PAGE. CDK4 and cyclin D1 protein were detected by immunobloting with specific antibodies. Values are the mean ± SD from three independent experiments. **P < 0.01 versus control.

Table 1. The effect of SPV and FPV on cell cycle (n=3, means ± SD).

Cell line	Dose (mg/ml)		Cell cycle (%)		
			G0/G1	S	G2/M
L1210	FPV	0	60.6	21.6	17.7
		0.02	70.2 ± 3.23**	16.4 ± 1.37**	13.3 ± 0.82**
		0.05	76.3 ± 4.47**	13.9 ± 1.01**	9.8 ± 1.13**
Hela	SPV	0	56.31 ± 2.77	21.51 ± 1.76	22.18 ± 3.02
		0.05	62.92 ± 3.46**	15.73 ± 1.9**	21.35 ± 1.24
		0.1	69.45 ± 3.39**	10.22 ± 1.37**	20.33 ± 2.08
MCF-7	SPV	0	56.5 ± 2.58	22.42 ± 3.28	21.08 ± 2.73
		0.05	60.82 ± 2.17**	18.86 ± 2.54**	20.32 ± 1.72
		0.1	64.16 ± 3.08**	15.26 ± 1.86**	20.58 ± 2.33
B16	SPV	0	58.33 ± 3.47	17.45 ± 0.98	24.22 ± 1.82
		0.05	61.56 ± 2.23**	13.55 ± 0.66**	24.89 ± 2.87
		0.1	65.13 ± 1.46**	10.73 ± 1.76**	24.14 ± 0.89

*P < 0.05, **P < 0.01 versus control

growth of L1210 cells in a dose-dependent manner (P<0.01). Compared with FPV, SPV showed very significant antiproliferative effects on the other three cell lines, especially on the Hela and B16 cells. Results of flow cytometric analysis also confirmed that FPV and SPV reduced cell viability with the same trend as MTT assay results. This cell type-selective inhibitory effect provided evidence that FPV may have chemopreventive potential on hematopoietic system malignancies (lymphocytic leukemia cancer) and SPV on solid tumors (melanoma, breast cancer, epithelial cervical cancer). Furthermore, it is likely that FPV and SPV have their respective compounds with actives anti-hematopoietic system malignancies and anti-solid tumors, and these results are much more favorable on bioactivity-guided isolations of FPV and SPV.

To further elucidate the mechanism of antiproliferative effects of FPV and SPV on malignancies, other methods for detection were used. A number of investigators observe that the G0/G1 cell cycle arrest and/or the S-phase fraction decrease correlated well with apoptosis (Massague, 2004). Our data confirmed that the cells treated with the *P. villosa* extracts, both FPV and SPV, exhibited statistically significant block of G0/G1 or G2/M phases, and obviously decrease the S-phase fraction (Table 1). Since cyclins and cyclin-dependent kinases regulate cell cycle progression, the expression of cyclin D1 and CDK4 in cancer cells were determined.

Cellular proliferation follows an orderly progression through the cell cycle, which is regulated by protein complexes that composed of cyclins and cyclin-dependent kinases. Cyclins are a family of cell cycle control proteins that regulate cell cycle progression by associating with and activating CDKs (Besson et al., 2008; Musgrove et al., 2011). The deregulated expression of G1 or G1/S phase cyclins or their related CDKs may cause loss of cell cycle control and thereby contribute to neoplastic transformation, because the major regulatory events leading to mammalian cell proliferation and differentiation occur in the G0 to G1 phases or in the G1 to S phase transition during the cell cycle (Harbour et al., 1999; Hartwell et al., 1994; Hunter et al., 1994). The Kaplan–Meier analysis show that cyclin D1 and CDK4 over expression are significantly associated with disease-free survival and overall survival, and these proteins over expression may play a pivotal role in the biological behavior of malignancies and may provide a strong prognostic implication (Dong et al., 2001). The results showed that SPV and FPV have similar effects in decreasing the protein expression of cyclin D1 and CDK4 protein. These results showed that SPV and FPV might inhibit cyclin D1 and CDK4 protein expression to inhibit tumor growth.

Conclusively, pharmacological data obtained from this study suggested that FPV and SPV possessed cancer chemopreventive potential on different types of cancer cells. The mechanisms involved in cancer chemoprevention by FPV and SPV extracts were cell cycle arrest and induction of apoptosis. A bioactivity-guided approach based on different type of cell lines growth inhibition will be taken to identify the active compounds in FPV and SPV.

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Full Length Research Paper

Intervention study on Chinese medicine Tongxinluo for cardiomyopathy in diabetic rats

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The aim of this study was to observe the effect of early application of the Chinese Medicine Tongxinluo on cardiomyopathy in diabetic rats. 36 rats were randomly divided into normal control group, diabetic group and Tongxinluo (TXL) group. Streptozotocin was used for preparation of diabetic model of Sprague Dawley (SD) rats. Tongxinluo powder (1.43 g of crude drug/g dry powder) was dissolved in water and used for gavage at a dosage of 0.3 g kg^{-1} day^{-1}. Blood glucose was detected 8 weeks later; left ventricular systolic pressure and left ventricular end diastolic pressure, maximum rise and fall rate of left ventricular pressure (± dp/dt max) were determined; myocardial pathological changes were observed under light microscopy and electron microscopy after Hematoxylin and eosin (HE) staining. ± dp/dt max in the diabetic rat group decreased, but ± dp/dt max of the Tongxinluo group increased, and the difference was significant ($P < 0.05$). Under light microscope, the myocardium myofibril of the diabetic group was deranged, and myofilament composition was reduced. Electron microscopy showed that cardiac endothelial cells were markedly swollen, and the basilar membrane of capillary vessel showed irregular obvious thickening. The lesions of the Tongxinluo group were mild. Tongxinluo can significantly improve cardiac function of diabetic rats and reduce diabetic cardiomyopathy.

Key words: Tongxinluo, diabetic rats, diabetic cardiomyopathy.

INTRODUCTION

Diabetic cardiomyopathy (DCM) refers to a disease state in which a wide range of structural abnormalities is caused by primary cardiac myocyte injury in patients with diabetes, ultimately leading to left ventricular hypertrophy, diastolic and (or) systolic dysfunction (Hayat et al., 2004), and is one of the major complications of diabetes, and is one of the main reasons leading to the death of patients with diabetes (Laakso, 1999).

Since Rubler et al. (1972) first proposed the diabetic cardiomyopathy, results of clinical, epidemiology, pathology and experimental studies all showed the presence of specific cardiomyopathy, instead of blood supply disorder secondary to coronary. Early diabetic cardiomyopathy may be asymptomatic, as the disease progresses, patients may have angina, arrhythmia and progressive cardiac dysfunction, due to the complexity of its causes but it also has its own pathophysiological change, so there are certain difficulties in clinical research and treatment. Therefore, exploration of the pathogenesis of diabetic cardiomyopathy and taking effective measures to control the occurrence and development of diabetic cardiomyopathy are of important significance to improve the quality of life of patients with diabetes and reduce its clinical mortality.

Diabetic cardiomyopathy is of significant influence on the prognosis of diabetic patients, therefore, to strengthen the study on its prevention and control is of great significance for the prevention of diabetic cardiovascular events. Chinese medicine has some advantages in prevention of diabetes and its complications, but there are still many problems in the current study, the focus of most research is still limited to lower blood glucose; studies on diabetic cardiomyopathy is lagging behind and is still limited to the description of the phenomenon, and the study on mechanism is far from in-depth research.

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Traditional Chinese medicine Tongxinluo can significantly improve myocardial ischemia and not only protect the integrity of the structure and function of small blood vessels, and its cardiac microvascular protective effect is more obvious. As a result, we conceptualized that the pathological changes of the Diabetic cardiomyopathy (DCM) are also caused by extensive microvascular change, prevention and treatment of DCM cardiomyopathy with Tongxinluo may have the same effect. This experimental design, with the use of the effect of traditional Chinese medicine Tongxinluo on DCM, is aimed to provide a theoretical basis for the seeking of effective drug treatment for the prevention and treatment of DCM, to make Tongxinluo have a new and broader prospect.

In this study, with the use of Tongxinluo for intervention in streptozotocin (STZ) diabetic rats, the effect of Tongxinluo on structure and function of the myocardium of diabetic rats was observed to ascertain the myocardial protective effect of the drug, so as to provide a theoretical basis for clinical treatment.

MATERIALS AND METHODS

Reagents

Streptozotocin (STZ) was purchased from Sigma (USA). The Tongxinluo ultrafine powder provided by Hebei Yiling Pharmaceutical Co., Ltd. (China) is mainly composed by ginseng, leeches, scorpion, eupolyphaga, centipedes, borneol, etc.

Animals and experimental groups

Male Sprague Dawley (SD) rats, aged 7 to 8 weeks, weight of 180 to 220 g, were provided by Liaoning Medical Experimental Animal Center. The 40 SD rats were randomly divided into two groups: 28 rats were taken and injected with 1% of STZ which was temporarily prepared with 0.5 mmol/L sterile citrate-citrate sodium buffer (pH 4.5) by a single injection in the tail vein of the rats at a dosage of 45 mg/kg; another 12 rats were used as the control group (control, CON) and injected with the same volume of the above buffer. Blood was taken from tail vein 72 h after the injection for blood glucose determination, rats with blood glucose concentration greater than 16.7 mmol/L were deemed as a model of diabetes. Four rats with blood glucose lower than this value was discarded, and then the diabetic rats (n = 24) were randomly divided into two groups: the diabetic group (DM), and Tongxinluo group (TXL). Rats of each group were reared in the cages of standard conditions, and reared for 8 weeks with standard diet (provided by Liaoning Medical Experimental Animal Center), and the Tongxinluo intervention group was given the Tongxinluo powder (1.43 g of crude drug/g dry powder) soluble in water at a dosage of 0.3 g kg^{-1} day^{-1} by gavages. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Liaoning Medical University.

Blood glucose determination

Tail blood was drawn from rats in each group before sacrificed, the glucose oxidase-peroxidase method was used, and the SureStep (steady doubly type) blood glucose monitoring device produced by Plus Johnson & Johnson and blood glucose test strips were used for blood glucose determination.

Determination of cardiac function

The rats were anesthetized with 20% of urethane (0.5 ml/kg body weight), catheter was intubated through the left ventricular cavity to the apex, the other end of the catheter was connected to a pressure transducer to record the left ventricular pressure curve (LVP), the LVP electrical signal was input into the Pclab biological signal acquisition and processing system to measure heart rate, maximum rise and fall rate of left ventricular pressure (± dp/dt max), left ventricular systolic pressure (LVSP) and Left ventricular end diastolic pressure (LVEDP).

Myocardial paraffin slicing and staining with HE

After sacrificing the rats in each group, tissue blocks from left ventricle was taken, fixed with 4% neutral formalin, dehydrated with graded ethanol, embedded in paraffin, and conventionally prepared into myocardial paraffin slices with a slice thickness of 5 μm, then stained with HE, and the slices were sealed with neutral gum.

Preparation of ultrathin myocardial slices and observation under TEM

Two rats in each group were randomly selected, anaesthetized with 20% urethane by intraperitoneal injection, then left ventricular myocardial tissue blocks were taken and cut into tissue blocks of 1 mm^3, and the ultra small specimens were placed into 3% glutaraldehyde fixative for fixation of 2 h, then fixed in 1% osmium tetroxide for 1 h, and then dehydrated with alcohol and acetone step by step, embedded with epoxy resin, and ultra-thin slices were sliced, with a slice thickness of 50 nm, after double stained with uranium and lead, the myocardial ultrastructural changes were observed with JEOL1200EX TEM scope. 10 electron microscope images of rat heart muscle capillaries (100 × 200 power) were taken from each group. The basement membrane cross-sectional area was the area of interest. 10 points at each capillary were selected for basement membrane thickness measurements. Means were calculated.

Statistical analysis

The SPSS11.5 statistical software was adopted for data processing and analysis, all measurement data were expressed as mean ± standard deviation (mean ± SD), the single factor analysis of variance (SNK method for q test), and $P < 0.05$ was for significant difference and $P < 0.01$ was for highly significant difference.

RESULTS

General state of the rats

At 2 days after injection of STZ, most rats showed diabetic symptoms such as polyuria, polydipsia, polyphagia, slow body weight gain, accompanied by various degree of listlessness, sparse and lackluster fur; four rats with blood glucose lower than 16.7 mmol/L were

Table 1 Effect of Tongxinluo on blood glucose and heart function of STZ diabetic rat ($\bar{x}$ ±s)

Group	Rat(n)	blood glucose (mmol/L)	-dp/dtmax (mmHg/s)	+dp/dtmax (mmHg/s)	LVSP	LVEDP
CON	12	9.2±4.56	3688.01±375.27	3870.54±386.18	126.66±7.56	3.74±0.36
DM	10	29.5±2.21	2718.05±255.90*	2907.27±148.86*	110.52±11.03	5.24±0.42**
TXL	10	25.4±3.25	3000.35±133.96	3168.36±166.63	112.60±11.59*	4.69±1.05*

Note:*p<0.05 **p<0.01 verse CON group

discarded, four rats died during feeding, and the general state of the TXL group was better than that of the DM group.

Effect of tongxinluo on blood glucose

After injection with STZ, blood glucose levels of the rats significantly increased and always fluctuated at a high level during the experiment; when fed for 8 weeks, blood glucose levels (29.5 ± 2.21) mmol/L of rats in the DM group were significantly higher than that (9.2 ± 4.56) mmol/L in the CON group, blood glucose (25.4 ± 3.25) mmol/L of the TXL group was at much higher levels, and showed no significant difference as compared with that of the DM group (Table 1).

Effect of tongxinluo on heart function of STZ diabetic rat

Heart function of the experimental rats can be determined by measuring left ventricular ± dp/dt max, LVSP and LVEDP, and changes in ± dp/dt max, LVSP and LVEDP, respectively reflected systolic dysfunction and diastolic dysfunction. When compared with the CON group at duration of 8 weeks, the ± dp/dt max of the DM group reduced for all rats, and the difference was significant; the LVEDP increased more significantly, and the difference was highly significant; for the TXL group, LVSP increased, but LVEDP decreased, and the difference was significant (Table 1).

Myocardial morphological changes of rats

Cardiac muscle of the CON group was arranged in order; for rats in the DM group, cardiac myocyte showed hypertrophy, myocardial myofibril was deranged, myofilament components reduced, and the gap between was noticeably wider; lesions of the TXL group were milder than that in the DM group (Figure 1).

Effects of tongxinluo on the myocardial ultrastructure

Under electron microscope, it can be seen that the capillary vessel basilar membrane of the cardiac muscle of rats in CON group was continuous and complete; the capillary vessel basilar membrane of the cardiac muscle of rats in DM group was significantly thickened, endothelial cells were significantly swollen and broke into the lumen in a "hump" like manner; thickening of the capillary vessel basilar membrane of the cardiac muscle of rats in TXL group was significantly reduced. The capillary basement membrane thickness of the normal control group (115 ± 10 nm) < that of the TXL group (148 ± 22 nm) < that of the DM group (247 ± 28 nm) (all $P < 0.01$) (Figure 2).

DISCUSSION

Diabetic cardiomyopathy (DCM) is a specific condition which is primarily manifested by left ventricular remodeling and diastolic and/or systolic dysfunction (Shankar et al., 2007). The primary pathological changes of DCM include the hypertrophy and apoptosis of myocardial cells, myocardial fibrosis, and myocardial microvascular extensive endometrial lesions (Okoshi et al., 2007), which lead to a decrease in myocardial compliance. These pathological changes can be fundamentally ascribed to hyperglycemia-caused glucose and lipid disorders. As an independent risk factor, hyperglycemia can directly cause myocardial damage and ultimately, lead to DCM (Nielsen et al., 2002).

DCM is an independent cardiomyopathy, has been recognized as a unique cardiac complication of diabetes mellitus (DM), and is closely related to the high incidence and high mortality of cardiovascular disease in diabetic patients. Early DCM show no obvious clinical manifestations, ventricular diastolic dysfunction can be found by color Doppler echocardiography, ventricular systolic dysfunction will gradually occur with the progress of the disease until heart failure. More than 90% of clinical diabetic patients were with type 2 diabetes (T2DM), and recent studies have shown that, in type 2 diabetes patients, in whom blood glucose was well controlled, 60% of the individuals still had diastolic dysfunction, thus study on CM is of important practical significance (Jayasankar et al., 2004).

Battiprolu et al. (2010) found that chronic hyperglycemia results in glucose toxicity, causing damages to cardioblasts, cardiac muscle fibers, and vascular

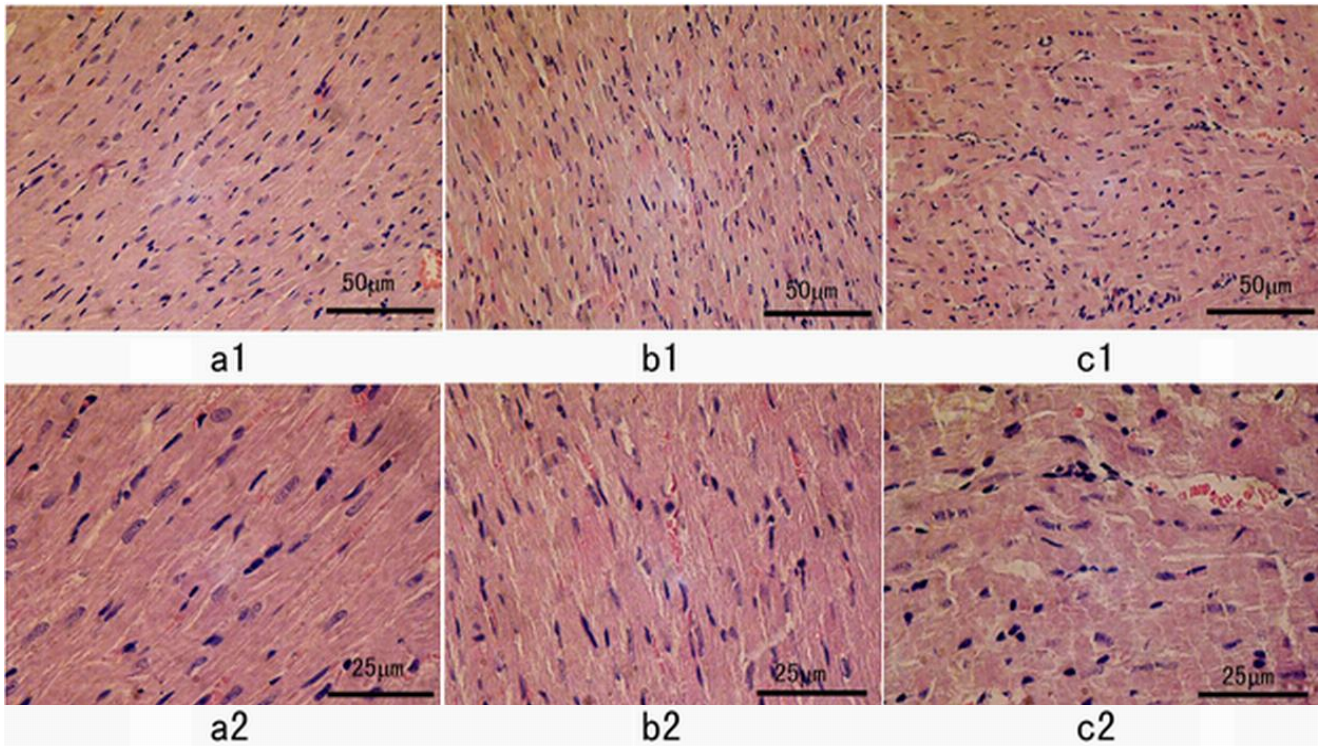

Figure 1. Myocardial morphology changes of rats in each group under light microscope. A1: HE stained CON group (200×); A2: HE stained CON group (400×); B1: HE stained TXL group (200×); B2: HE stained TXL group (400×); C1: HE stained DM group (200×); C2: HE stained DM group (400×).

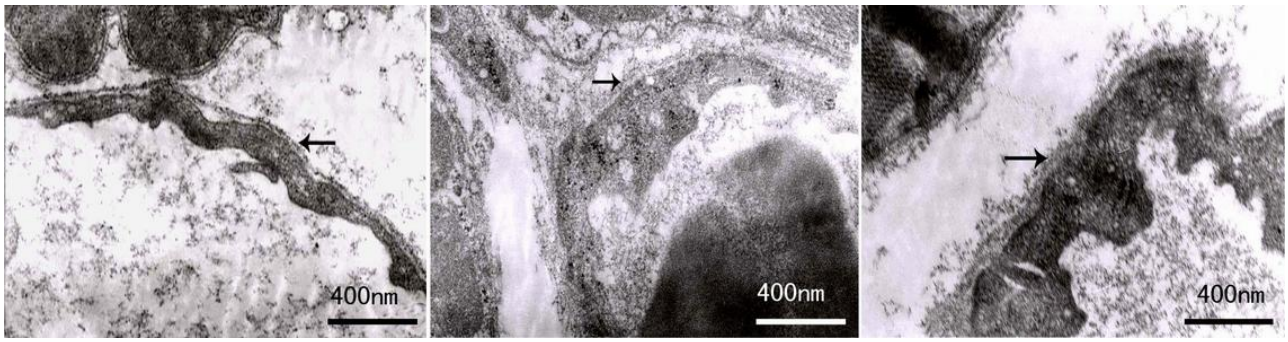

Figure 2. Effects of Tongxinluo on the myocardial ultrastructure of STZ diabetic rats. A: electronic microscope CON group (20000×); B: electronic microscope TXL group (20000×); C: electronic microscope DM group (20000×). Note: → marked in electron microscopic observation refers to the capillary vessel basilar membrane.

endothelial cells. Wang et al. (2006) reported that terminal glycosylation end products (AGEs) are one of the mechanisms underlying DCM and that they lead to myocardial fibrosis and cardiac dysfunction.

Diabetic cardiomyopathy is one of the main reasons leading to the death of diabetic patients, therefore, to slow down the pathological process and to improve the prognosis are of important clinical significance in reducing mortality. In this experiment, STZ induced diabetic rat models were established, Tongxinluo was applied for intervention treatment, and was aimed to observe the structural and pathological changes in cardiac muscle and changes in cardiac function in diabetic rats as well as the effect of Tongxinluo on diabetic cardiomyopathy.

Effect of tongxinluo on the cardiac function of rats with STZ-induced diabetes

DCM triggered by a metabolic disorder can cause myocardial cytological changes, and thus subclinical heart dysfunction will occur and later progress to small

vessel disease of myocardial muscle, microcirculation disorder and cardiac autonomic neuropathy, and eventually lead to cardiac dysfunction (Shama and Mcneill, 2006). An early manifestation of diabetic cardiomyopathy is decline of left ventricular diastolic function (Fischer et al., 2003). In DM patients, the accumulation of myocardial collagen and deposition of excessive advanced glycation end products (AGE) will result in decrease of left ventricle elasticity and diastolic dysfunction (Asif et al., 2000). Cardiac Doppler examina- tion revealed that, except for type 2 diabetes patients associated with hypertension and coronary heart disease, approximately 60% of the patients showed reduction in left ventricular diastolic function (Poirier et al., 2001). Studies by Karamitsos et al. (2007) found that, before the occurrence of ventricular systolic dysfunction in diabetic patients, left and right ventricular diastolic dysfunction has already occurred, and this change may be related to interventricular interdependence and the uniform and consistent action of diabetes on the two ventricles.

The results in this experiment suggest that, after injection of STZ, blood glucose was significantly increased, rats showed diabetic symptoms such as polyuria, polydipsia, polyphagia. At eight weeks of course, rats in the DM group showed both cardiac diastolic and systolic function abnormalities, which were manifested as more obvious diastolic dysfunction, and -dp/dt max were significantly reduced; ± dp/dt max in the TXL group was significantly increased, indicating that Tongxinluo improved the diastolic dysfunction of diabetic rat hearts. As reported in literature, changes of cardiomyocytes cardiac function in diabetic rats induced by STZ occurred within 6 to 14 weeks (Thompson, 1988).

This is consistent with our observations. Recently, it is found that diabetic left ventricular diastolic dysfunction is related to the duration of diabetes and hyperglycemia (Henry et al., 2004). Noninsulin-dependent diabetes, hypertension, microalbuminuria or proteinuria, cardiovascular events, and ramipril (DIABHYCAR) studied and observed congestive heart failure in diabetic patients over 50 years old, and found that the basic glycosylated hemoglobin (GHb) level is a predictor of the further development of congestive heart failure (Vanr et al., 2003).

In patients with Type 1 diabetes accompanied by interventricular septal thickening and left ventricular mass increase, with reduction in GHb, interventricular septal thickness and left ventricular mass can be recovered to a certain extent (Aepfelbacher et al., 2004). Studies by Iribarren et al. (2001) have shown that, the degree of impaired diastolic function is related to glycosylated hemoglobin, and the main mechanism is that, advanced glycation end products combined with collagen macromolecules and gathered, which stimulates the release of fibroblast growth factors, resulting in the increase of inflammatory reactions of myocardial cells. It is also reported that, the increased thickness of ventricular in patients with type 1 diabetes can be partially reversed one year later by strictly controlling glucose, which relieved the nosogenesis that blood glucose has been involved in diabetic cardiomyopathy (Grandi et al., 2006).

Effect of tongxinluo on the cardiac function structure of rats with STZ-induced diabetes

A large number of studies have found that, high blood glucose can lead to myocardial hypertrophy and myocardial fibrosis (Narasimman et al., 2005). Diabetic cardiomyopathy is manifested as deposition of positive materials of myocardial microvascular periodic acid-Schiff reaction (PAS), thickening of the vascular basilar membrane, perivascular fibrous tissue hyperplasia, occlusion and dilatation of myocardial microvascular stenosis (Takai and Miyazaki, 2002). Microvascular disease is one of the important reasons for development of diabetic cardiomyopathy. Recent studies have found that, myocardial collagen deposition and myocardial fibrosis may be an important factor inducing diabetic cardiomyopathy (Way et al., 2002), and its pathophysiological changes are of its own uniqueness.

By continuous observation of the diabetic model animals, it is found that, myocardial fiber hypertrophy, deposition of Periodic acid-Schiff (PAS) positive material among myocardial fibers, focal necrosis of myocardial fibers, fatty degeneratiaon of myocardial cells, thickening of basilar membrane of myocardial capillary vessels, endothelial cell hyperplasia accompanied by mamillary prominency, subendothelial fibrous tissue proliferation, deposition of PAS positive material within the vessel wall, transparent thrombosis within endovascular lumen and vascular stenosis or even occlusion was observed. Travelling disorder of cardiac myofibril can be observed under electron microscope, with different directions of bending, forming several small branches, muscle sections of varying lengths, cystic dilatation of the sarcoplasmic reticulum, myofilament dissolution, formation of myeloid bodies, swelling, limited increase and dissolution of mitochondria deposition of glycogen granules, and significant thickening of capillary vessel basilar membrane.

It was reported in literature that, at 8 weeks after induction with STZ, cardiomyocytes of diabetic rats have shown typical pathological changes of diabetic cardiomyopathy, such as cardiac hypertrophy, hyperplasia, interstitial edema and ultrastructural changes (Zhu et al., 1993). It is observed in this experiment that, under light microscope, cardiac cell hypertrophy, dearrangement of cardiac myofibrils, reduction of myofilament composition, significant widening of interstice can be observed in rats of DM group; significant thickening of the capillary vessel basilar membrane of the cardiac muscle, significant swelling of endothelial cells and their breaking into the

lumen in a "hump" like manner can be observed in rats in the DM group under electron microscope.

In TXL group, dearrangement of cardiac myofibrils, widening of interstice, thickening of the capillary vessel basilar membrane of the cardiac muscle and swelling of endothelial cells was significantly reduced as compared with that of rats in DM group, therefore, it can be concluded that, Tongxinluo has certain protection effect for cardiac muscle of diabetic rats. For the mechanism of protection function of Tongxinluo for heart, it is believed by clinical studies that, it may mediate the protection effect by increasing nitrogen monoxide (NO) level, to inhibit serum endothelin (ET). Animal experiments show that, Tongxinluo can reduce myocardial infarct size after ischemia reperfusion, reduce the level of plasma creatine kinase (CK), and thus has protection effect for ischemia and reperfusion cardiac muscle.

Tongxinluo is constituted by leech, scorpion, eupolyphaga, centipede, cicada, ginseng, red peony root and borneol, and is of effect of supplementing qi and activating blood circulation, as well as removing obstruction in channels to relieve pain. Ginseng has the effects of invigoration, benefiting qi, regenerating body fluid, regulating immunity, and so on. Ginseng total saponin promotes the aerobic oxidation of glucose, enhances the activity of succinate dehydrogenase, as well as promotes insulin release to some extent. Red peony roots and Eupolyphaga seu steleophaga can decrease blood fats, guard against arteriosclerosis and platelet aggregation, and improve blood hypercoagulability. Furthermore, they can remove oxygen radicals in tissues, inhibit mitochondrial swelling and lipid peroxidation, as well as protect mitochondrial structures and functions. Leeches, scorpions, *Periostracum cicadae*, scolopendra, and *Borneolum syntheticum* can promote blood circulation for removing blood stasis, eliminate inflammation and relieve pains, dredge channels and collaterals, dispel wind, and improve immunity, and so on. This study show that Tongxinluo can significantly improve the heart function of diabetic rats, and can reduce diabetic cardiomyopathy, and its mechanism may be by protecting the microvascular, to prevent diabetes progress to diabetic cardiomyopathy. To date, although some findings concerning the application of Tongxinluo in the prevention and cure of DCM have been obtained, most are obtained from animal experiments. Therefore, to provide wider application prospect for Tongxinluo for this use, a large scale clinical studies remain to be carried out. Furthermore, the specific action mechanisms underlying the effect of Tongxinluo on myocardial preservation remain to be explored.

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Full Length Research Paper

Proximate and nutrient analysis of selected medicinal plants of Tank and South Waziristan area of Pakistan

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Inhabitants of Tank and South Waziristan area of Pakistan are facing acute shortage of medicines and food. The purpose of this study was to evaluate the medicinal plants of the area for their nutrient and medicinal values and to recommend their preservation/propagation for medicinal and/or food purposes. The plants investigated were *Alhagi maurorum* Medik, *Datura alba* Ness, *Chenopodium album* L. *Tecomella undulata* (Sm.), *Withania coagulans* Dunal, *Berberis lycium* Royle. Proximate parameters like protein, fat, fiber, carbohydrates, moisture contents, ash, and energy values were obtained using Association of Official Analytical Chemists (AOAC) methods. Macronutrients (Ca, Mg, Na, and K) and micronutrients (Fe, Cu, Zn, Cr, Cd, Pb, and Ni) were analyzed by employing atomic absorption spectrophotometer. The study showed that *Datura album* has higher nutrient value than *Withania coagulans* and there exist a significant correlation among the results. Further, the plants were found to be useful for medicine and food purpose.

Key words: Proximate analysis, protein, fiber, macro- and micro-nutrients, medicinal plants, Tank, South Waziristan area.

INTRODUCTION

Market is full of synthetic drugs having high prices, severe side effects and affecting the environment whereas medicinal plants and the drugs derived from them are cheaper in cost, have lesser side effects and hence popular among the people (Alfawaz et al., 2006). According to a survey, 75 to 80% of the world's population relies over such plants (Atta et al., 2004) as they are famous for healing several diseases and are considered as a healthy source for life (Ndubani et al., 1999; Verpoorte, 2000; Harvey et al., 2000). Though, Pakistan has biodiversity in climate and in geographical situation and has valuable medicinal plants heritage (Newman et al., 2000), but its flora is neither properly explored for medicinal point of view nor for food value (Shinwari et al., 2000); in spite of the facts that medicinal plants are considered to be mostly rich in nutrients (Farnsworth, 1994). On the other hand, the dwellers are victim of malnutrition and facing tremendous problems due to economic position of people (Siddiqui et al., 2000), non-availability of standard drugs and food stuff in the area (Pandey et al., 2006; Pieroni et al., 2000).

The prevailing situation has provoked us to analyze the bioactive plants with reference to micro and macronutrients and their food values (Shinwari et al., 2004; Hussain et al., 2010). The following plants were selected for analysis based on their utility by natives as medicines and their reported bioactivity. *Chenopodium album* is antipyretic (Dai et al., 2002), antinociceptic (Kumar et al., 2007), sperm immobilizing agent (Gohar et

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Table 1. Species collected for study with local name and families.

Species name	Family name	Local name	Parts used	Status
A. maurorum	Fabaceae	Thanda	Whole plant	Wild
D. alba	Solanaccae	Badalbangae	Seed/Whole plant	Cultivated
C. album	Amaranthaceous	Batoo	Seed/Leaves	Cultivated/Wild
T. undulate	Bigoniaceae	Rohida	Seed/Bark	Wild
W. coagulans	Solanaceae	Paniry poda	Seed/Whole PLANT	Cultivated/Wild
B. lyceum	Berberidaceae	Kashmal/Ishkeen	Fruit/Roots bark	Wild

al., 1997), and hypertensive and is rich in iron contents (Yadav et al., 2002). *Datura alba* used in asthma, muscle spasm, whooping cough, hemorrhoids, skin ulcer, anesthetic for setting bones, bruises and menstruation (Chithra et al., 1998; Satyavati et al., 1976). *Tecomella undulata* has antifungal (Azam et al., 1999), antitermite (Ahmad et al., 1994), analgesic and anti-inflammatory property (Budhiraja et al., 1984). It is used for the cure of syphilis, eczema and relaxant, cardio tonic and chloretic activities (Batanouny, 1999). Its leaves have oleanolic acid, ursolic acid and betulinic acid, compounds that are strong in prohibiting HIV (Dushyent et al., 2000). Traditionally, *Alhagi maurorum* is used for gastrointestinal disorders, gastric ulcer and rheumatism (Khushbaktovaet al., 1992). *Withania coagulans* is used for the treatment of diabetes mellitus (Abouzid et al., 2010) and have antibacterial, antifungal (Hemalatha et al., 2008), anti-inflammatory (Choudhary et al., 1995), antitumor cardio-vascular activity (Gaind et al., 1967; Khan et al., 1993). *Berberis lyceum* is well known for its anti-inflammatory and immune-potentiating property (Gupta et al., 2008). The berbamine inhibits hepato-carcinogenesis and possesses anticancer activity (Gilani et al., 1992).

The current study has been desined to analyze proximate composition and mineral profile of *Alhagi maurorum, Datura alba, Chenopodium album, Tecomella undulata, Withania coagulans and Berberis lyceum*.

MATERIALS AND METHODS

Plants collection

The plants were collected from Tank and South Waziristan region of Khyber Pukhtoon Khawa province, Pakistan from March to May, 2008 and were identified by Prof. Dr. Muqarab Shah, Chairman, Department of Botany Hazara University, Mansehra. Specimen of each plant was deposited in the Herbarium of Botany Department, Peshawar University, Peshawar, KPK Pakistan. The collected plant species, their family, botanical and local names are listed as shown in Table 1.

Proximate and chemical analysis

Each collected plant sample was dried under the shade and was finely ground using an electric grinding machine (Model MX 491N, National) to raw flour separately. The analysis was then made using standard techniques provided by Association of Official Analytical Chemists (AOAC, 1990).

The moisture contents were determined by drying the sample at 105°C in the oven up to constant weight. The crude protein value of the sample was assessed by determining the total organic nitrogen using Micro-Kjeldahl's apparatus (Komel et al., 2000). The crude lipids were extracted in petroleum ether at 40 to 60°C, using Sox-let apparatus, and then evaporating the solvent up to dryness using evaporator (Pandey et al., 2006). For the estimation of the fiber contents, the dry outcome of lipid estimation was ignited and the ash contents were determined and taken as equivalent to fiber contents (Hussain et al., 2010). Carbohydrate contents of each sample were calculated using the difference method as follows:

Carbohydrate (%) = 100 - (moisture (%) + protein percentage (%) + lipid (%) + ash contents (%))

Whereas, the energy values of each sample were determined using the following formula.

K calories/100 g = 9 (crude fats (%)) + 4 (carbohydrates (%) + proteins (%)).

Elemental analysis

The plant was ignited to ash and the ash was dissolved in HCl to bring the ash in solution form. The macro- and micronutrients were then determined using single beam atomic absorption spectrometer provided by Perkin Elmer, USA (Bibi et al., 2006).

Statistical analysis

Proximate and elemental analysis of each plant sample was carried out thrice for each parameter and the mean, standard deviation and standard error were calculated. Inter-element correlation was performed using Statistical Package for Social Sciences (SPSS V.14).

RESULTS AND DISCUSSION

The proximate compositions and calorific values calculated over dry weight of the samples are displayed in Table 2. The moisture contents of the samples were 5.55% in *B. lycium* and 14.22 % in *D. alba.* Carbohydrates contents were the highest among all the investigated parameters and were from 32.35 (*W. coagulans*) to 92.65% (*C. album*) (Table 2). The low concentration of crude fat and ash was recorded in *T. undulata* and in *W. coagulans*; while the high contents were in *D. alba* and *C. album* (Table 2). High value of protein and fiber were

Table 2. Nutritional values of selected medicinal plant species*.

Species	M (%)	A (%)	P (%)	F (%)	F (%)	C (%)	EV (Kcal/100 g)
A. maurorum	8.76 ±0.01	12.66±0.02	6.56±0.02	4.88±0.01	3.33±0.01	56.52±0.12	330.51±0.01
D. alba	14.22±0.02	6.58±0.00	12.10±0.19	16.49±0.01	9.21±0.09	65.64±0.06	290.40±0.21
C. album	9.13±0.31	21.15±0.03	15.21±0.00	3.92±0.02	7.58±0.07	92.65±0.02	420.92±0.30
T. undulate	7.73± 0.01	4.52±0.03	9.44±0.06	2.52±0.11	18.3±0.01	74.08±0.06	380.39±0.01
W. coagulans	6.82 ±0.09	2.32±0.01	4.51±0.02	8.24±0.00	8.85±0.02	32.35±0.03	261.33±0.19
B. lycium	5.55± 0.00	7.75±0.01	7.67±0.03	5.32±0.01	13.5±0.20	46.99±0.23	485.70±0.35

*Values are the mean ± standard deviations of triplicate determination: M=Moisture, A=Ash, P=Protein, F=Fat, C=Carbohydrate, EV=Energy values.

Table 3. Correlation matrix of proximate parameters.

Parameter	Correlation coefficient	P-value (extent of interdependency)	Status
Moisture vs. ash	0.13	0.81 (>0.05)	Non-significant
Moisture vs. protein	0.54	0.26 (>0.05)	Non-significant
Moisture vs. fat	0.76	0.07 (>0.05)	Strongly positively correlated
Moisture vs. fiber	-0.29	0.57 (>0.05)	Non-significant
Moisture vs. carbohydrates	0.39	0.44 (>0.05)	Non-significant
Moisture vs. energy value	-0.46	0.35 (>0.05)	Non-significant
Ash vs. protein	0.67	0.15 (>0.05)	Non-significant
Ash vs. fat	-0.31	0.55 (>0.05)	Weakly negatively correlated
Ash vs. fiber	-0.49	0.35 (>0.05)	Non-significant
Ash vs. carbohydrates	0.70	0.11 (>0.05)	Non-significant
Ash vs. energy value	0.42	0.40 (>0.05)	Non-significant
Protein vs. fat	0.15	0.84 (>0.05)	Non-significant
Protein vs. fiber	0.03	0.96 (>0.05)	Weakly positively correlated
Protein vs. carbohydrate	0.91*	0.01 (<0.05)	Significant
Protein vs. energy value	0.32	0.54 (>0.05)	Non-significant
Fat vs. fiber	-0.23	0.66 (>0.05)	Non-significant
Fat vs. carbohydrate	-0.21	0.66 (>0.05)	Non-significant
Fat vs. energy value	-0.59	0.24 (>0.05)	Moderately negatively correlated
Fiber vs. carbohydrate	0.08	0.89 (>0.05)	Non-significant
Fiber vs. energy value	0.32	0.45 (>0.05)	Non-significant
Carbohydrate vs. energy value	0.35	0.50 (>0.05)	Non-significant

found in *T. undulata* (18.32%) and *C. album* (15.21%), while the low contents were found in *W. coagulans* (4.51%) in *A. maurorum* (3.33%). The highest calorific value was recorded in *B. lyceum* (485.70 Kcal/100 g) followed by *C. album* (420.92 Kcal/100 g) and *W. coagulans* (261.33 Kcal/100 g) (Figure 2). The samples were found to be a good source of carbohydrates and to some extent of protein. The values obtained for all investigated parameters are in agreement with the values recorded in Microsoft Encarta Premium DVD 2009. Similar studies have also reported previously by (Irvine 1992, Naseem et al, 2006, Zia-Ul-Haq et al., 2007, 2011, 2012; and Nisar et al, 2009). The results obtained of protein, fat, fiber, carbohydrates, moisture contents, ash, and energy values for Withania coagulans (Hussain et al., 2010) Datura alba (Hussain et al., 2011) quite different from the present result obtained. This is may be due to environmental condition. The other species nutritionally evaluated for the first time.

Elemental analysis of the aforementioned six medicinal plants showed significant variation among macro- and micronutrients (Table 4 and Figure 1). In case of macronutrients out of all the six reported species, *D. alba* showed the highest Ca contents (6329 mg/kg), while *T. undulata* (1321 mg/kg) stood lowest. Similarly, the highest concentration of Mg (45460 mg/kg), K (14991 mg/kg) and Na (895 mg/kg) were found to be in *C. album, A. maurorum* and *B. lyceum,* respectively, while the lowest were found in *A. maurorum* (1292 mg/kg), *W. coagulans* (2450 mg/kg) and *T. undulata* (91 mg/kg). The

Table 4. Concentration of macro- and micro-nutrients of selected medicinal plant species.

Species	Ca	Mg	K	Na	Fe	Cu	Zn	Cr	Cd	Pb	Ni
A. maurorum	2234	1292	14991	650	105.4	14.3	8.5	2.5	0.2	0.7	2.5
D. alba	6329	20248	13535	190	80.2	8.3	95.8	4.6	1.5	2.6	1.4
C. album	4242	45460	10455	375	102.8	4.3	0.2	1.8	0.6	0.00	0.3
T. undulata	1321	4021	3840	91	18.2	16.4	6.4	0.7	0.9	0.3	0.00
W. coagulans	9260	35280	2450	125	98.8	2.2	40.2	0.6	1.4	1.9	1.8
B. lyceum	4621	3240	2640	895	76.2	0.6	25.5	2.8	2.1	0.4	2.2

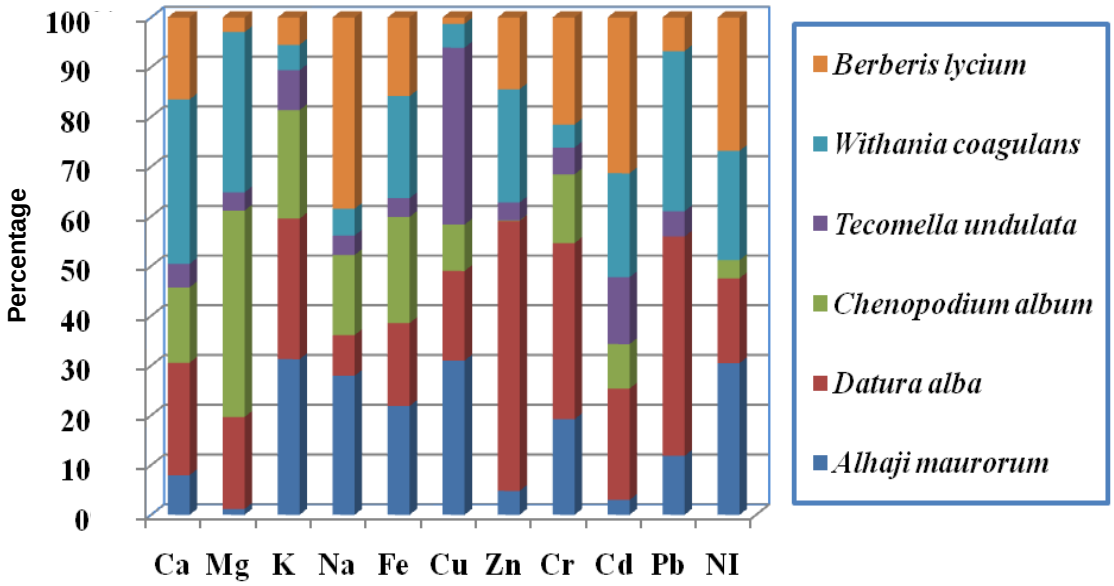

Figure 1. Summary of the macro- and micro-nutrients analysis of the medicinal plants.

results obtained for micronutrients analysis showed that the concentration level of Fe is extremely high in *A. maurorum* as compared to *T. undulate.* In the case of Cu, it was the highest in *T. undulata* followed by *A. maurorum.* Zn contents were the highest in *D. alba* (95.8 mg/kg), followed by *W. coagulans* (40.2 mg/kg) and *Berberis lycium* (25.5 mg/kg), which is in the expected range (25 to 150 mg/kg) (Chopra et al., 1986). The other three species (*A. maurorum, C. album* and *T. undulata*) had below the stated range. The contents of Cr were below the toxic (10 mg/kg) level in all the species, while the contents of Cd, Pb, and Ni were negligible (Dastagir et al., 2004) (Table 4).

Moisture, ash, protein, fat, fiber, carbohydrates contents and energy value have also been determined in the investigated species. We simply calculated the bi-variable correlation co-efficient using the average for a replication of three observations (Table 3), and tried to develop relationship between significance and non-significance value. It has been observed that there exists very strong correlation between carbohydrates and protein with a relationship value of 0.91. It can therefore be strongly concluded that species having high contents of carbohydrate will also have high protein value. On the other hand, moderately negative correlation up to the extent of 0.57 was observed between fat and energy (Table 5).

Conclusions

Six medicinal plants, *A. maurorum, D. alba, C. album, T. undulata, W. coagulans and B. lyceum* were investigated for their proximate analysis. The obtained results showed that *B. lyceum* and *D. alba* had the highest carbohydrates contents among the investigated plants. The low contents of crude fat and ash were in *T. undulata* (2.52%), *W. coagulans* (2.32%), *D. alba* (16.49%) and in *C. album* (21.15%). The high value of protein and fiber were in *C. album* (15.21%) and in *T. undulata* (18.32%) while the low contents were in *W. coagulans* (4.51%) and in *A. maurorum* (3.33%). The highest calorific value was recorded in *B. lyceum* (485.70 Kcal/100 g). These results concluded that the plants are good source of

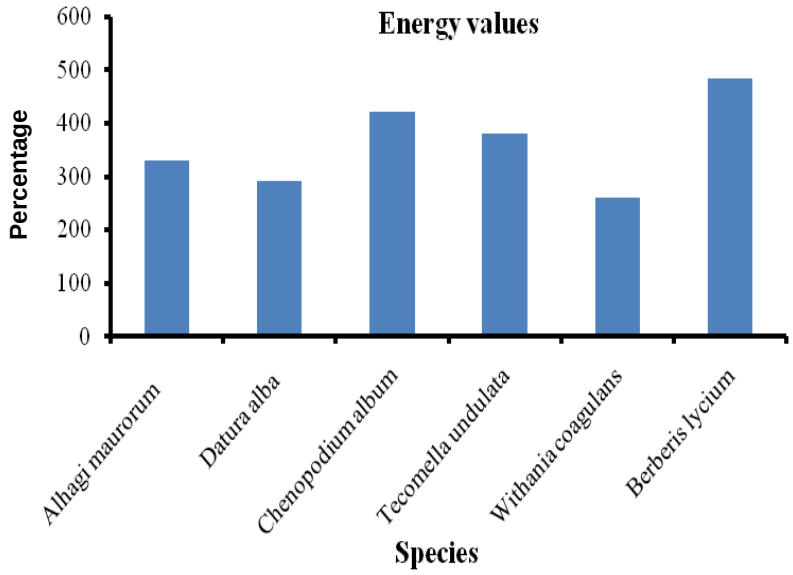

Figure 2. Showing energy value content in different plant species.

Table 5. Descriptive statistics.

Parameter	Mean content	Standard deviation
Moisture	8.71	3.00
Ash	9.16	6.82
Protein	9.25	3.89
Fat	6.89	5.06
Fiber	10.14	5.17
Carbohydrate	61.37	21.12
Energy value	361.50	83.97

carbohydrates and nutrients and up to some extent proteins.

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Full Length Research Paper

Descriptive study of contemporary status of the traditional knowledge on medicinal plants in Bulgaria

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In the contemporary reality of globalization and urbanization in Bulgaria, traditional empiric ethnobotanical knowledge is disappearing. The aim of our study was to check the attitude of a random sample of people to the herbs and the traditional way of healing: 1) to find out which herbs are the most popular in Bulgaria; 2) what is their most popular application; 3) to study the distribution of negative or positive attitudes to the traditional use of medicinal plants according to age and gender of Bulgarian people. In total, 77 plant species (including the ones in the formula combinations) were mentioned during the study. They belong to 38 families. The most important families are Lamiaceae, Rosaceae, Asteraceae and the plants which most commonly referred to as "Granny's cure" were *Hypericum perforatum*, *Cotinus coggigria, Plantago major, Sempervivum* sp. div., *Calendula officinalis, Melissa officinalis, Allium sativum, Aesculus hippocastanum*, *Matricaria chamomilla* and *Cornus mass.* The greatest number of herbs were the ones used to treat disorders of central nervous system (CNS), bones, skin, gastro-intestinal and respiratory system.

Key words: Traditional healing, medicinal plants, remedial properties.

INTRODUCTION

In the contemporary reality of globalization and urbanization in Bulgaria (worldwide tendency), traditional empiric ethnobotanical knowledge is disappearing. More often we find that when we question rural people they say, "There used to be a woman**,** who knew all about medicinal plants**,** but she passed away". The traditional experts belong to both sexes, but the great majorities were women. Few of the women experts were famous but many of them had a good general knowledge of the subject. This is not surprising**,** because the mother is, usually, the parent who would deal with most common ailments and illnesses. Despite that discouraging tendency observed in ethnobotanical researches during the last decade, we have found several examples of traditional empiric data which had not been documented and so we were inspired to see what more had been overlooked.

As a whole, the traditional knowledge about medicinal plants and their usage, preserved and transmitted from generation to generation, is quite well documented in Bulgaria. The collecting of common names was pioneered by teachers, University professors, naturalists, folklorists and physicians during 19 and 20th centuries. These pioneers recorded their use for conventional remedial purposes and also their use in traditional spells and magical rituals (Stanev, 2010). This collection of ethnobotanical data led to the publication of valuable scientific works (Petkov, 1982; Mitrev and Popova, 1982; Kitanov, 1987; Pamukov, 1992; Nikolov, 2006). The traditional knowledge documentation bases the list of 741 taxa recognized in Bulgaria by the law as medicinal plants, although the therapeutic effect and application is not specified (Medicinal Plants Act, 2000). The formulas and recipes of the famous Bulgarian healer, Peter

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Dimkov, are well known for their efficacy, and are accepted by the vast majority of Bulgarians. His books have gone into numerous editions, the last one after his death (Dimkov, 2001). The resent decades ethnobotanical research has been performed both by national and foreign scientists (Ivancheva and Stantcheva, 2000; Leporatti and Ivancheva, 2003; Ploetz and Orr, 2004; Kültür and Sami, 2009; De Boer, 2010).

The aim of our study was to investigate the attitude of a random sample of people to the herbs and the traditional way of healing: 1) to find out which herbs are the most popular among the population of Bulgaria; 2) what is their most popular application; 3) to study the distribution of the negative or positive attitudes to the traditional use of medicinal plants according to the age and gender of Bulgarian people. The study has a preliminary character and will reveal the critical points in the contemporary situation that need to be investigated in more details.

MATERIALS AND METHODS

The most common method used in ethnobotanical studies is interviewing potential respondents. The focus of the interviews may be a certain therapeutic category or a wider, less defined scope. The author's assessment of the implications of the study, and of the further uses of a particular medicinal plant may, of course, differ from one to another (Martin, 1995; Alexiades, 1996; Nolan and Robbins, 1999; Ertug, 2000; Cunningham, 2001; Vandebroek et al., 2004; Everest and Ozturk, 2005; Pieroni et al., 2005; Heinrich et al., 2009; Pieroni et al., 2011; Anderson et al., 2011; Mustafa et al., 2012).

In order to test the current state of traditional knowledge about medicinal plants, we performed the study as a Rapid Ethnobotanical Appraisal. It forms in the way of a structured interview based on fixed questions concerning plants used for certain health disorders. The interviews were conducted in a short time without requiring expensive tools because the participants sought to obtain sketch of local conditions rather than an in-depth-study. A small group of local people was selected and interviewed qualitatively about a wide range of topics in a semi-structured way, allowing a comprehensive view of how the community behaved as a whole. The interviews are highly visual and are carried out by community members, often in collaboration with the researcher (Gerique, 2006).

Our research team consisted of University lecturers in pharmaceutical botany and pharmacognosy and undergraduate students specially trained for the purpose of the study. We devised a questionnaire listing some of the main groups of medicinal problems. In order to trigger the informants and obtain as much as possible information without boring and repelling them**,** we tried to balance between not enough detailed and too heavy list. Some informants are prone to explain more details than it is possible to explore dealing problems with vision, inflammation of the eyes, ears, skin, rash, warts, joint pains, rheumatism, sciatica, exostoses, failures of the immune system, colds, bronchitis or other problems of the respiratory tract, contraception, miscarriage, breast feeding, mastitis, colics, bedwetting, blood disease, hypertension, heart disease*,* gastrointestinal disorders (diarrhoea and constipation), kidney and urinary tract problems (cystitis and prostatitis), menstrual disorders, treatment of trauma/wounds, memory loss and insomnia.

We were looking mainly for information flow that passed from generation to generation in a verbal way and the stories start with motto "Granny's cure for this was ….".

During the summer period of 2011, 183 interviews were carried out. This is a preliminary study that we intend to conduct in depth in the future. Target groups were the few people**,** that we knew to be particularly interested in medicinal plants and traditional ways of healing but the majority was a random sample of people – male and female of different social status and different ages, above 18. As we aimed to get a Rapid Ethnobotanical Appraisal on the contemporary status of the traditional knowledge about medicinal plants in Bulgaria**,** the interviews were performed in towns and villages from different districts of Bulgaria (Figure 1). As a result of urbanization**,** strict topographic localization of the knowledge is impossible. The data obtained from the inhabitants of a town might come from local villages just as often as distant ones**,** because of the processes of migration.

In this study, the plants were identified according to Jordanov (1963-1995). Ideally, we obtained a sample of the plant in question, or 'voucher material', but most often we were given a description corresponding to the common name. Voucher material presented for identification was deposited in the Herbarium at the Faculty of Pharmacy, Medical University Sofia. If the voucher was in bad condition it was rehydrated in warm dilute alcohol, prepared correctly so that diagnostic features remained visible, and dehydrated carefully. Then it was either identified by comparison to reference material in the herbarium (registered Herbaria of the University of Sofia and the Institute of Botany) or we consulted the leading taxonomist in the taxonomically problematic group of Dr. M. Anchev (*Brassica* sp.). In this second case, fruits were required for correct identification so we could not go further that the genus level.

The collected data are shown in Table 1. The plants were listed according to the number of cases in which they were mentioned for a particular therapeutic effect**.**

The analyses were performed with the methods of the non-parametric statistics as the data do not belong to any particular mode of distribution and they have a ranking but no clear numerical interpretation. Analyses were as follows: 1) systematic of medicinal plants that we found to be popular among the target groups; 2) health disorders grouping; 3) share between persons who are interested in traditional use of healing plants versus indifferent and negative ones. The distribution according to age and gender was also analyzed.

RESULTS AND DISCUSSION

What herbs were currently most popular among the population of Bulgaria? – Systematic of medicinal plants

Totally, 77 plant species (including the ones in the formula combinations) were mentioned during the investigation. They belong to 38 families (Figure 2 and Table 1). The most important families were Lamiaceae, Rosaceae and Asteraceae as their members were among the most often mentioned medicinal plant species, respectively 9 members of the first two families and 6 members of the third family. The families Apiaceae, Brassicaceae, Ericaceae, Fabaceae and Ranunculceae were popular of their 3 members each.

Most popular Granny's cure plants were *Hypericum perforatum*, *Cotinus coggigria, Plantago major* and *Sempervivum* sp. div.; *Calendula officinalis, Melissa officinalis, Aesculus hippocastanum, Matricaria chamomilla* etc (Table 1).

Figure 1. Districts of Bulgaria where interviews were performed.

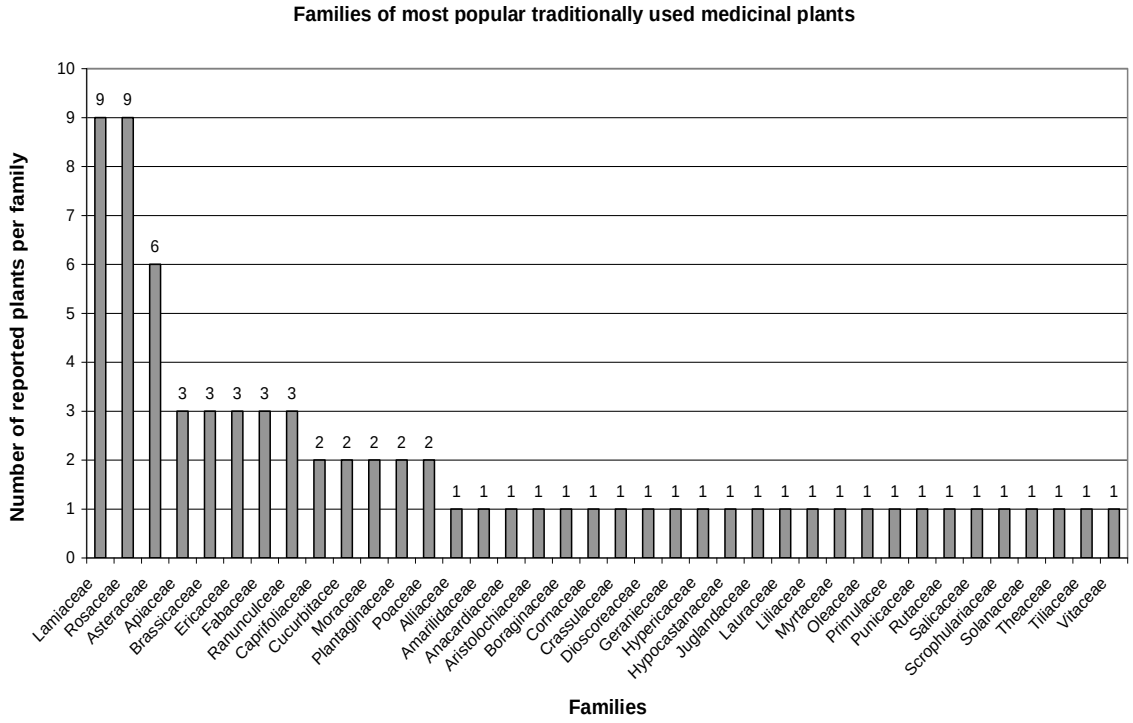

Figure 2. Systematic of medicinal plants currently popular as “Granny’s cure” – frequency of reports of plant family members.

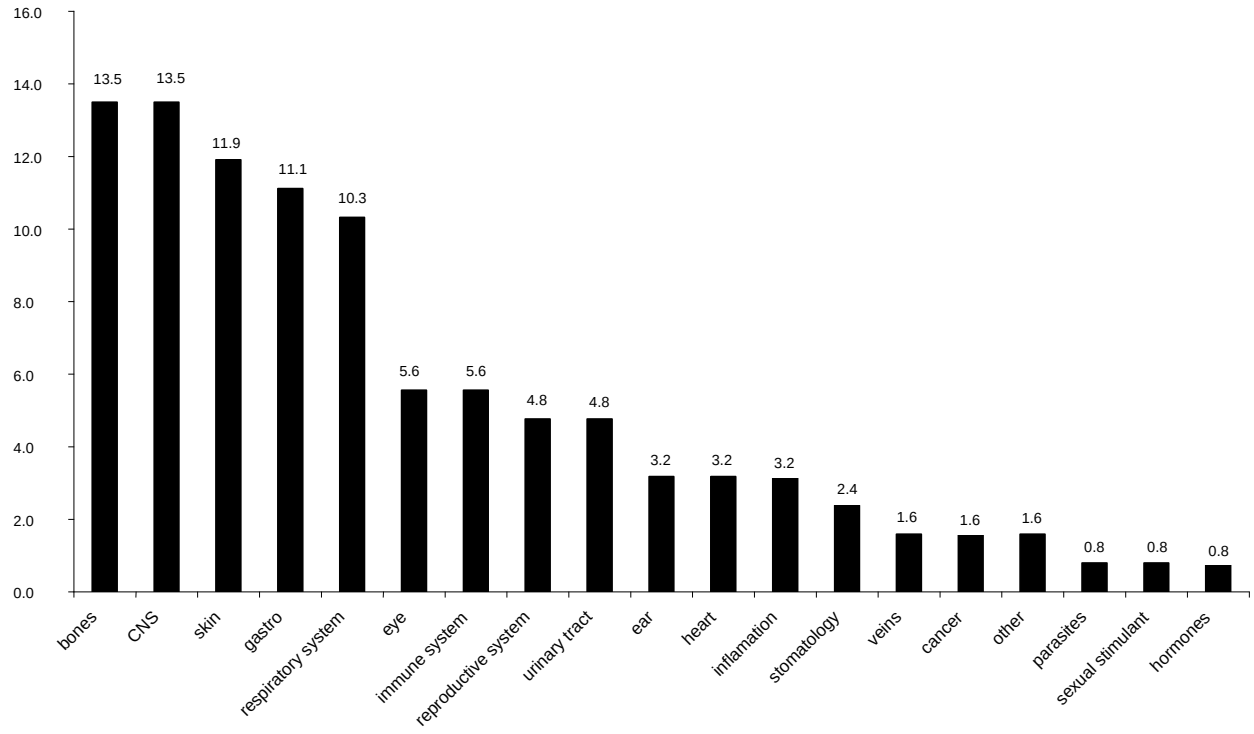

Figure 3. Health disorders grouped by systems, treated traditionally with plants.

What was most the popular application and for which health disorders?

Most herbs are used to treat disorders of central nervous system (CNS) and bones with 17 reports for both of them, followed by those of skin - 15, gastro-intestinal - 14, respiratory system - 13 reports etc. (Figure 3). Within the system disorders, we distinguish subgroups of disorders. The most often mentioned conditions treated with medicinal plants are respectively exostoses and rheumatoid/joint pain; wounds, injuries, pustule, pus; diarrhea, gastritis, gastric ulcer, colitis; and coughs and lymph nodes inflammation. As we aimed to record most precisely the way informants shared their knowledge, some quite similar disorders fall in different subgroups within the main system group (Figures 4 to 6 and Table 1).

The respondents report that each condition might be treated with number of plants species. For instance, exostoses are treated with *Allium cepa, Allium sativum*, *Ononis spinosa*, *Pulsatilla pratensis, Sambucus ebulus, Tamus communis, Neium oleander, Salix* sp. div., *Brassica oleracea.* Rheumatoid/joint pains are treated with *Sinapis arvensis, A. hippocastanum, C. coggigria, Aloe vera, Eucaliptus* sp. Insomnia is treated with *M. officinalis, Achillea millefolium, Tilia* sp. div., *Mentha piperita*. Hypertonia is treated with *Geranium macrorhyzum, Allium schoenoprasum, Olea europaea* and *M. officinalis* (Table 1).

Usually**,** application was mentioned as pure plant substances. In sporadic cases, combination of 1 to 7 herbs formulas was shared. For example**,** *Astragalus glycyphyllos* (herba), *Teucrium polium* (herba), *Capsella bursa-pastoris* (herba), *M. officinalis* (herba), *M. piperita* (folium), *O. spinosa* (radix) and *Betula pendula* (folium) are used as decoction in case of sterility. Combination of *Juglans regia* (fructus – fruit shells or entire fruits), *A. cepa* (bulbus) and *Cydonia oblonga* (seed) prepared as a decoction is applied against cough. Another traditional decoction formula are *Solanum tuberosum* (tuber), *Tilia sp* div. (inflorescentia), *C. oblonga* (fructus), *A. cepa* (bulbus) and *Malus domestica* (fructus). The last one is applied against cough in vulnerable and delicate babies' and in early childhood.

The application that we registered is basically relevant to the records that have been published for Bulgarian traditional and official medicine which relays on medicinal plants (Petkov, 1982; Mitrev and Popova, 1982; Pamukov, 1992; Dimkov, 2001; Nikolov, 2006). Some of the well known data initiate modern detailed research like for instance the antinociceptive effect of *M. chamomilla* (Nouri and Abad, 2012).

We found some new information that has not been recorded yet. *Thalictrum minus* L. (Ranunculaceae) was reported to have quite rapid wound healing effect and reduction of scars. The plant is so far known to contain berberine-type alkaloids and has cytostatic effect (Kumazawa et al., 1984; Velcheva et al., 1992; Vanisre

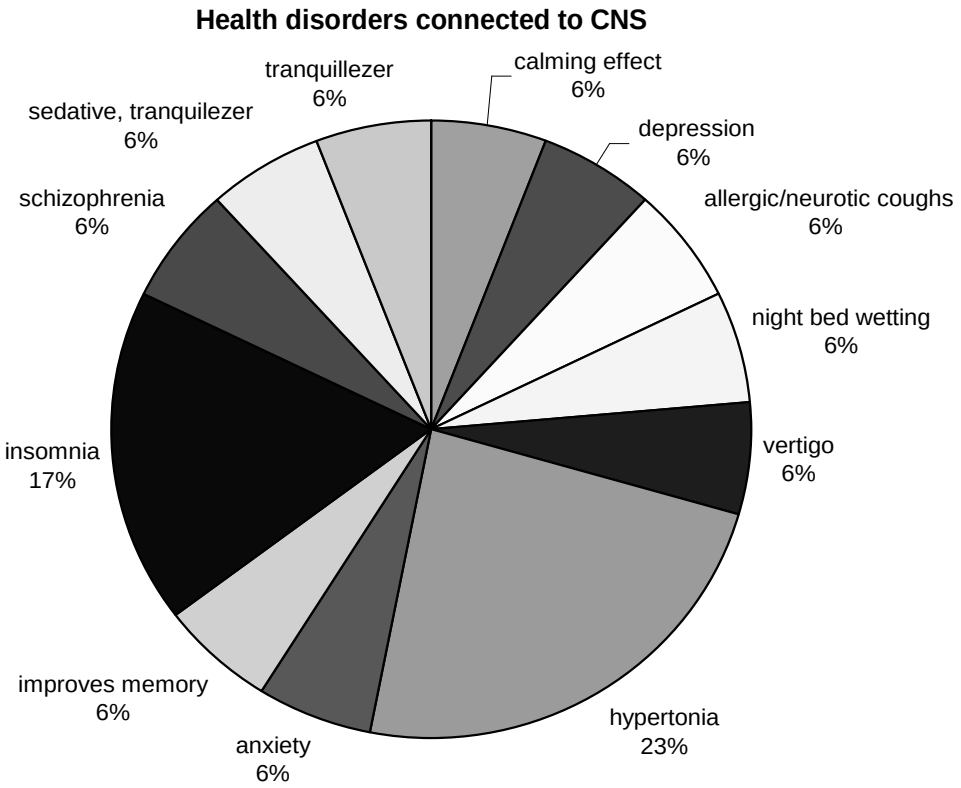

Figure 4. Health disorders connected to CNS treated traditionally with plants in the way they were reported by informants.

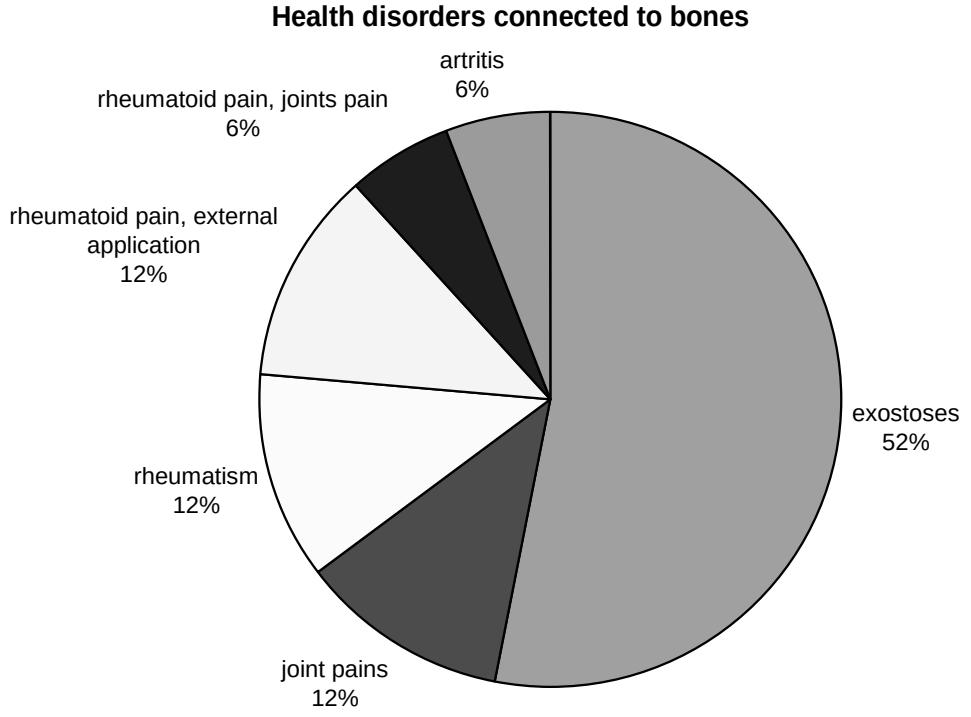

Figure 5. Health disorders connected to bones treated traditionally with plants in the way they were reported by informants.

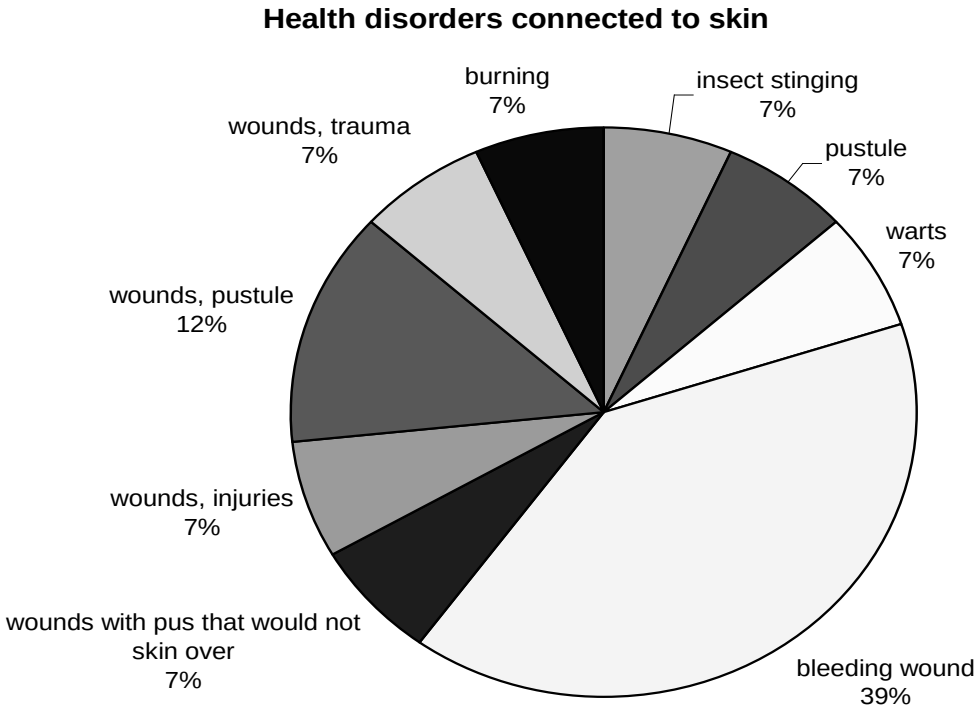

Figure 6. Health disorders connected to skin treated traditionally with plants in the way they were reported by informants.

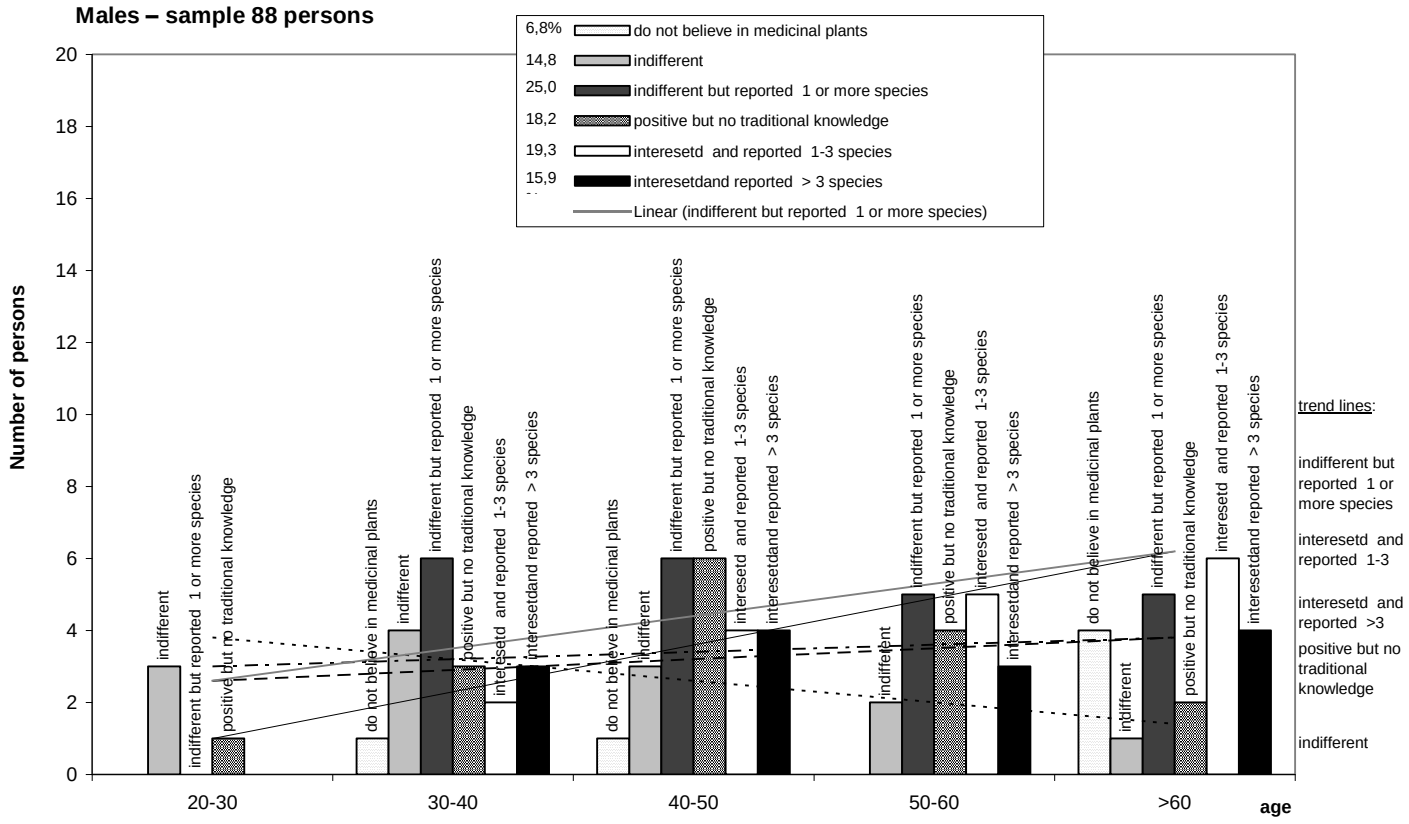

Figure 7. Attitude to the traditional use of medicinal plants amongst the male informants.

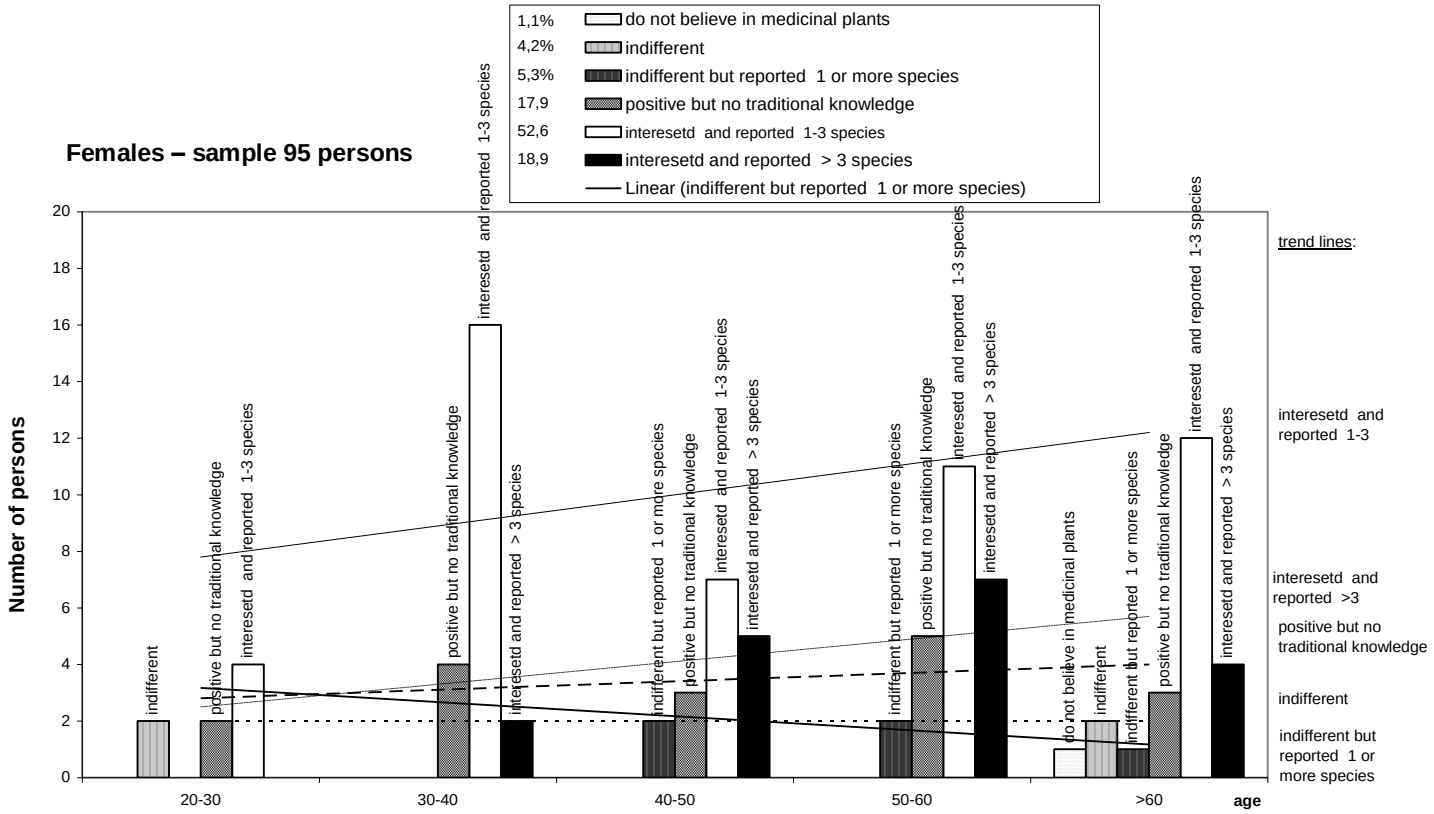

Figure 8. Attitude to the traditional use of medicinal plants amongst the male informants.

et al., 2004). *Pulsatilla vulgaris* Mill. (Ranunculaceae) was reported that fresh leaves are used as compress against exostoses. Warning that in case of prolonged application "muscles could be melted" was given too. The plant so far is known as sedative and anaphrodisiac (Petkov, 1982). It is prescribed as central-acting analgesic (Yarnell, 2002). In homeopathy *Pulsatilla* 6X is used against migraine disorders, vertigo, neuralgic pain, venous stasis (Gottwald and Weiser, 2000).

Demographic analysis – how negative or positive attitude to the traditional use of medicinal plants was related to age and gender of people?

Although we tried to keep the gender and age balance of the informants from the 183 questioned persons, 48% (88) were males, 52% (95) were females. The slightly prevalent age group was between 40 and 50 years for men (27.3%) and between 50 and 60 years for women (26.3%).

"Do not believe in medicinal plants" or "indifferent" was the response of 21.6% of the males. Positive and more or less knowledgeable were 78.4% of the males. "Do not believe in medicinal plants" or "indifferent" was the response of only 2.3% of the females. Positive and more or less knowledgeable were 94.7% of the females (Figures 7 and 8).

Most of the male informants (25%) said they were not interested in healing plants, but reported one or more species they know. The tendency of this attitude was to increase with the age of persons (Figure 7).

Most of the female informants (52.6%) said they were interested in medicinal plants and familiar with 1 to 3 species. The basic tendency of this attitude was to increase with the age of persons with a peak and the highest percent (27.1%) in the age group between 30 and 40 years old (Figure 8). Within this age group the women who were interested in medicinal plants and familiar with 1 to 3 species consisted 72.8%. Possible explanation to this fact is the motherhood and the desire to treat the children with natural methods when possible.

Conclusion

As a whole, the Bulgarians are open to traditional methods of healing. Our study would have positive impact to the practice to define rational combinations between traditional and conventional treatment approaches. Main implications for further research should be ethno and social pharmacy investigations on the

Table 1. Currently popular as "Granny's cure" medicinal plants among the population of Bulgaria, used plant's parts and application presented in the way the knowledge was shared by informants. Note: sometimes the information given by people differs from the officially recognised activity.

Plant species	Used part	Form of use	Health disorders	Pure/combination	Reports
Achillea millefolium L.	Herba	Decoction	Hearth disorders	Pure	1
	Herba	Decoction	Dysmenorrhoea, hyper menorrhoea	Pure	2
	Herba	Decoction	Insomnia	Pure	1
A. hippocastanum L.	Semen	Spiritus extract	Joint pains	Pure	7
	Semen	Spiritus extract	Varicose veins	Pure	5
Agrimonia eupatorium L.	Herba	Decoction	Sour throat	Pure	1
A. cepa L.	Bulbus	Juice in the nostrils	Cold, running nose	Pure	4
	Bulbus	Compress	Exostoses	Tar	2
Allium porrum	Herba	Fried in sunflower oil	Ear inflammation	Pure	5
A. sativum L.	Bulbus	Fried in sunflower oil	Ear inflammation	Pure	2
	Bulbus	Juice applied externally	Exostoses	Pure	1
	Bulbus	Juice applied externally	Insect stinging	Pure	4
	Bulbus	Internal application	Cold	Pure	6
A. schoenoprasum subsp. *sibiricum* (L.) Syme	Folium	Fresh - internal application	Hypertonia	Pure	1
A. vera Mill.	Folium	Juice applied externally	Rheumatoid pain, joints pain	Pure	1
Apium graveolens L.	Folium	Decoction	Arthritis	Pure	1
Arctostaphylos uva-ursi (L.) Spreng.	Folium	Decoction	Kidney disorders	Pure	2
Aristolochia clematitis L.	Herba	Decoction	Aphrodisiac (warning for highly toxic)	*Eupatorium cannabinum* L.	1
A. glycyphyllos L.	Herba	Decoction	Sterility	*T. polium* L. (herba), *C. bursa-pastoris* (L.) Medik. (herba), *M. officinalis* L. (herba), *M. piperita* L. (folium), *O. spinosa* L. (radix) *Betula pendula* Roth (folium)	1
Brassica oleracea cultivar	Folium	Fresh leaves compress	Exostoses	Pure	4

Table 1. Contd.

	Folium	Fresh leaves compress	Rheumatism	Pure	5
	Folium	Raw, compress	Mastitis	Pure	2
Brassica sp.	Herba	Decoction	Colon tumours	Pure	1
C. officinalis L.	Herba	Ointment	Burning	Lard	1
	Anthodium	Decoction, compress	Mastitis	Pure	1
	Herba	Ointment	Wounds	Lard	10
	Herba	Ointment	Varicose veins	Lard	1
	Herba	Ointment	Cold	Lard	1
C. busa-pastoris (L.) Medik.	Herba	Decoction	Hyper menorrhoea	Pure	3
	Herba	Tinctura compress	Wounds, trauma	Pure	2
Carduus sp. div.	Flos	Decoction	Heart disorders	Pure	2
M. chamomilla L.	Anthodium	Decoction internally/inhalation	Throat pain, cold	Pure	7
	Anthodium	Decoction, compress	Swollen eyes	Pure	2
	Anthodium	Decoction, lavement	Cleansing face or genitalia	Pure	1
C. mass L.	Fructus	Fresh, canned or dry preserved	Diarrhoea	Pure	10
Cotinus coggygria Scop.	Folium	Decoction, lavement	Genitlia infections	Pure	2
	Folium	Compress fresh leaves/decoction	Wounds with pus that would not skin over	Pure	13
	Folium	Infusion, lavement	Lesions of the cervix	*J. regia* L.folium	1
	Folium	Spiritus extract	Rheumatoid pain, external application	Pure	1
Crataegus monogyna Jacq.	Folium	Decoction	Calming effect	*Mentha* sp. *Valeriana officinalis* L.	5
Cucurbita pepo L.	Semen	Raw	Anticestodes	Pure	6
C. oblonga Mill.	Fructus	Caned	Diarrhoea	Pure	2
	Fructus	Caned	Coughs	Pure	2

Table 1. Contd.

Cynodon dactylon (L.) Pers.	Rhyzome	Decoction	Cystitis	Pure	2
Daucus carota L.	Radix	Raw	Vision stimulant	Pure	1
Ecbalium elaterium A. Rich.	Fructus	Fresh	Sinusitis	Pure	2
Eucaliptus sp.	Folium	Spiritus extract	Rheumatoid pain, external application	Pure	3
Ficus carica L.	Latex	Fresh external application	Warts	Pure	7
Galanthus nivalis L.	Flores	Spiritus extract	Heart disorders	Pure	2
Geranium macrorhyzum L.	Folium	Fresh	Hypertonia	Pure	3
Helleborus odorus Waldst. & Kit. ex Willd.	Rhyzome	Compress	Inflamed tonsils	On strip of soft dough	1
H. perforatum L.	Herba	Decoction before meal	Gastritis, gastric ulcer, colitis, kidney disorders,	Pure	12
	Herba	Decoction	Anxiety	Pure	4
J. regia L.	Fructus	Syropus - unripe fruits	Thyroid gland	Pure	3
	Fructus	Decoction	Coughs - babies and kids	*A. cepa* L. (bulbus), *C. oblonga* Mill. (seed)	4
	Folium	Infusion	Glaucoma	*Morus alba* L., *Ficus carica* L.	1
Laurus nobilis L.	Folium	Decoction	CNS depression, allergic/neurotic coughs	Pure	1
Levisticum officinale W.D.J. Koch	Folium	Decoction	CNS depression	Pure	1
Mespilus germanica L.	Folium	Decoction	Diarrhoea	Pure	2
M. officinalis L.	Folium	Decoction	Sedative, tranquillizer	Pure	9
	Herba	Decoction	Hypertonia	Pure	2
	Herba	Decoction	Improves appetite	Pure	2
M. piperita L.	Folium	Infusion	Insomnia	Pure	6
	Folium	Infusion	Stomach pain	Pure	2
Mentha viridis (L.) L.	Folium	Decoction	Stomach pain, diarrhoea, nausea	Pure	3
Morus nigra L.	Fructuts	Fresh - locally applied	Aphthous stomatitis	Pure	2

Table 1. Contd.

Nerium oleander L.	Folium	Extract 96% spiritus	Exostoses	Pure	2
Nepeta nuda L.	Herba	Decoction - external	Mastitis	Pure	1
	Herba	Decoction - external	Wound	Pure	1
	Herba	Decoction - internal	Cystitis	Pure	1
	Herba	Decoction	Prostatitis	Pure	1
Olea europaea L.	Folium	Decoction	Hypertonia	Pure	1
O. spinosa L.	Radix	Decoction	Exostoses	Pure	1
P. vulgaris Mill.	Folium	Fresh/dry substance infusion	Aphthous stomatitis	Pure	1
P. major L.	Folium	Pate	abdominal tumours	Pure	1
	Folium	Raw, compress	Wounds, pustule	Pure	11
	Folium	Decoction	Stomach ulcer	Pure	3
Plantago subulata L.	Folium	Decoction	Schizophrenia	Pure	1
Potentilla reptans L.	Herba	Decoction	Mastitis	Pure	2
	Herba	Decoction	CNS night bed wetting	Pure	1
Primula veris L.	Folium	Infusion	CNS vertigo	Pure	1
Prunella vulgaris L.	Herba	Decoction	Haemorrhoids	Pure	1
Prunus domestica L.	Fructus	Raw or decoction	Constipation	Pure	4
P. vulgaris Mill.	Herba	Fresh leaves compress	Exostoses	Pure	1
Punica granatum L.	Fructus	Decoction (cortex)	Diarrhoea	Pure	3
Rosa sp. div.	Radix	Decoction	Diarrhoea	Pure	1
	Hips	Decoction, lavage	Immune system	Pure	5
	Hips	Decoction, lavage	Cystitis	Pure	1
	Galls	Decoction	Coughs of bronchial origin	Pure	2
Rubus idaeus L.	Folium/Fructus	Decoction	Fever	Pure	2
Ruta graveolens L.	Folium	Spiritus extract	Ear inflammation	Pure	1
Salix sp. div.	Radix	Decoction	Exostoses	Pure	2

Table 1. Contd.

Salvia officinalis L.	Herba	Infusion	Improves memory	Pure	1
Salvia verticillata L.	Folium	Fresh leaves compress	Wounds, injuries	Pure	1
Sambucus ebulus L.	Foliom	Immature fresh leaves compress	Exostoses	Pure	1
Sambucus nigra L.	Flos	Decoction	Coughs	Turiones Pini	2
Sambucus nigra/ebulus L.	Fructus	Juice	Immune system	Pure	3
Sempervivum sp.div.	Folium	Juice or the whole leaf	Ear inflammation	Pure	13
	Folium	Compress	Pustule	Pure	2
Sinapis arvensis L.	Semen	Pad	Joint pains	Pure	1
Solanum tuberosum	Tuber	Fresh, compress	Eye irritation or UV burn	Pure	2
	Tuber	Decoction	Coughs - babies and kids	*Tilia sp div.* (inflorescentia), *C. oblonga* Mill. (fructus), *A. cepa* L. (bulbus) *Malus domestica* Borkh. (fructus)	1
Stachys germanica L.	Folium	Raw, compress	Wounds	Pure	1
Symphytum officinale L.	Radix	Spiritus Extract	Rheumatism	Pure	1
Tamus communis L.	Radix	Spiritus extract	Exostoses	Pure	1
Taraxacum officinale F.H. Wigg	Radix	Infusion	Wounds	Pure	2
Teucrium chamaedrys L.	Folium	Decoction	Wounds	Pure	1
Thalictrum minus L.	Folium	Decoction	Wounds, pustule	Pure	1
Thea sinensis L.	Leaves	Infusion, lavage	A stye in the eye	Pure	1
Thymus serpilus	Herba	Decoction	Cold	Pure	4
	Herba	Decoction	Appetite stimulant	Pure	4
	Herba	Decoction	Hearth tranquilizer	Pure	4
	Herba	Decoction	Tranquilizer	Pure	5
	Herba	Compress	Lymph nodes inflammation	Brandy	1
Tilia sp. div.	Flos	Decoction	Fever	Pure	1
	Flos	Decoction	Insomnia	Pure	1

Table 1. Contd.

Trifolium pratense L.	Herba	Infusion	Cold	*Rubus idaeus* L.folium, Rose hips	1
Tussilago farfara L.	Folium	Fresh leaves compress	Wounds	Pure	1
	Folium	Decoction	Coughs	Pure	1
Vaccinium myrtillus L.	Fructus	Decoction	Vision stimulant	Pure	2
Vaccinium vitis-idaea L.	Fructus	Raw or decoction	Cystitis	Pure	1
Veronica chamaedrys L.	Herba	Decoction	Toothache	Pure	1
Vinca major L.	Folium	Infusion	Perfusion	Pure	1
Vitis vinifera L.	Tendril sap	Fresh	Vision stimulant/ cataract	Pure	4
Zea mays L.	Stigmata	Decoction	Cystitis, kidney disorders	Pure	4

potential consumption of medicinal plants and herbal medicinal products.

This is related to the necessity of trending the consumption of certain medicinal plants with limited resources. On the other hand**,** it is crucial to support marketing of some plants that are cultivated and additionally exploited.

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Full Length Research Paper

The effect of genistein for preventing granulose cell injury induced by cisplatin

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Genistein (GEN), the primary isoflavone in legumes, has a well known weak estrogenic effect by binding to estrogen receptors, and widely used for the treatment of ovary disease induced by chemotherapeutics, however, the details of the exact mechanisms was unclear so far, thus, the aim of our study was to find the effect on granulosa cells of ovary induced by cisplatin (CDDP) after using GEN treatment by (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) method and flow cytometry. The results demonstrated that CDDP could inhibit the proliferation of granulosa cells of ovary by affecting cell cycle S stage. Moreover, CDDP also could arrest cell cycle in G1-M stage, which would evidently increase the number of apoptotic cell. Genistein has the potential to prevent the damaging effect of CDDP and improve the differentiation and proliferations of the cells to make the blockage of the cell cycle disappear, which is related to the dose of GEN and time. The present study provides improvement in understanding the molecular pathogenic mechanism of premature ovarian failure (POF) induced by chemotherapeutics and development of GEN as effective treatment drugs.

Key words: Genistein, Premature ovarian failure, Granulosa cells of ovary, Flow Cytometry.

INTRODUCTION

Premature ovarian failure (POF) is a disorder of multicausal etiology, leading to infertility in women before the age of 40 (McGee and Hsueh, 2000; Rees and Purdie, 2006).). With cure rates of cancers in childhood and young women improving, it is likely that the incidence of prematurely menopausal women will rise rapidly (Sklar et al., 2006; Panay et al., 2008; Rebaret et al., 1990). There are more and more patients suffering from ovarian deficiency and infertility caused by chemotherapy, which becomes one of the important etiological factors of POF (Goswami and Conway, 2005; Krishna et al., 2010). The disease model is more complex and difficult to prevent with therapeutics, hence it is necessary to understand molecular mechanisms responsible for premature ovarian failure caused by chemotherapy and study new technologies and introduce new drugs to prevent and treat the POF.Genistein (GEN) is a phytoestrogen that occurs naturally in the diet and is found in a wide variety of plant-derived foods especially in soybeans and soy-based foods (Park et al., 2010). GEN has a well known weak estrogenic effect by binding to estrogen receptors (Kim et al., 1998). Increasing evidence showed that GEN plays a major role in prevention of cancer (Imhof and Molzer, 2008), osteoporosis (Taku et al., 2010), heart diseases (Sbarouni et al., 2007), and cognitive dysfunction (Thorp et al., 2009). GEN has recently received considerable research attention on the mechanism of their actions in ovarian disease. There is growing interest in the beneficial effects of GEN on postmenopausal symptoms (Suthar et al., 2001). Furthermore, GEN has been intensively investigated in recent years as a chemopreventive agent, mainly against hormonally regulated POF by

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chemotherapy in animal models (Huang et al., 2008). But additional studies are needed to elucidate the pharmacological mechanisms of GEN treatment for POF by chemotherapy.

In light of the existing, yet unresolved views on POF by chemotherapy, in this study, we used granulosa cells of ovary induced by cisplatin (CDDP) to explore the effect of GEN on proliferation of granulosa cells of ovary, which might help us to learn about the effects of GEN on granulosa cells of ovary cell inhibition at cellular level.

MATERIALS AND METHODS

Isolation and culture of granulosa cells of ovary

Immature female Wistar rats were obtained from animal experimental center of Beijing University of Chinese Medicine (China) at 21 days of age, housed in standard cages, and provided food and water *ad libitum*. All protocols were approved by the Institutional Animal Care from Beijing University of Chinese Medicine. Starting on day 22, some animals were injected twice daily with Pregnant mare's serum gonadotropin (PMSG) (Sigma, USA) for up to 48 h to induce follicular development. Animals were euthanized by inhalation of Halothane. Ovaries were aseptically removed to DMEM-F12 tissue culture medium . The ovaries were quickly trimmed of surrounding adipose tissue, bursa, and connective tissue. Primary cultures of rat granulosa cells were established as previously described (Clemens et al., 2000). Rat granulosa cell lines were cultured in DMEM-F12 containing 5% fetal bovine serum (Sigma, USA) until approximately 70% confluent and then used for assays.

MTT assay

The *in vitro* drug sensitivity to cisplatin was assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Granulosa cells were plated at a density of 50,000 cells in 96-well plates. They were allowed to recover for 72 h and then exposed to various concentrations of etoposide and carboplatin for 24 to 72 h. Then, drug cytotoxicity was evaluated by using a MTT reduction conversion assay (Sigma, USA). Forty microliters of MTT at 5 mg/ml concentration was added to each well, and incubation was continued for 4 h. The formazan crystals resulting from mitochondrial enzymatic activity on MTT substrate were solubilized with 200 µl of dimethyl sulfoxide, and absorbance was measured at 490 nm by using a SpectraMAX microplate reader (Molecular Devices, USA). Each combination of cell line and drug concentration was set up in eight replicate wells, and the experiment was repeated three times, then, half maximal inhibitory concentration of cancer cells (IC_{50}) was counted. Cell survival was expressed as absorbance relative to that of untreated controls. Granulosa cells of ovary induced by cisplatin using IC_{50} concentration was determined by MTT assay after treatment of at the concentration of 0.5×10^{-6}, 1×10^{-6}, 2×10^{-6}, 4×10^{-6} mol/L. The detail manuscript was performed according to the above experiment.

Flow cytometry

Confluent of granulosa cells of ovary were seeded in 12-well culture plates at a density of $5 \times l0^5$ cells/well. After serum-starvation for 24 h, granulosa cells were incubated with DMEM containing 2×10^{-6} mol/L of genistein and the IC_{50} concentrations of cisplatain for 96 h. The cells were detached from the plates using 0.1% trypsin, 1% EDTA and fixed with 70% ethanol. After two washes with PBS, granulosa cells were incubated with 1% Triton X-100 and 0.1 mg/mL RNase A at 37°C for 30 min. After centrifugation (1,200 rpm for 5 min at room temperature), the supernatant was discarded and 0.1 mg/mL propidium iodide (PI) was added, and placed on ice for 30 min. After passing through a nylon filter membrane, the cells were analyzed by flow cytometry to determine cell cycle. Each condition was analyzed in quadruplicate and the statistical package SPSS13.0 (SPSS Incorporated, Chicago) was used to analyse all group data.

Statistics analysis

The statistical package SPSS13.0 (SPSS Incorporated, Chicago) was used for all analysis. One-way ANOVA test was used to determine the significance of differences among the groups. All values were expressed as mean ± SD. In general, p values less than 0.05 were considered statistically significant.

RESULTS

Effects of GEN on cell viability

To determine the effects of cell viability and inhibition induced by cisplatin, the viability of the treated cells was measured by MTT assay. As shown in Table 1, significant cytotoxic effect and cell growth inhibition on cells was showed by cisplatin. Compared with control group, the viability of ovary cells exposed to h cisplatin was significantly inhibited with increasing time. In addition, the viability of ovary cells in high dose cisplatin were significantly increased compared to low dose cisplatin ($P < 0.05$) (Table 1).

IC_{50} of cisplatin was 8.85 µg/mL by calculation. MTT assay was also performed to determine the effects of genistein on cisplatin induced cell injury and viability. As shown in Table 2, high dose of GEN could significantly decrease cisplatin induced decrease in cell viability compared to low dose of GEN ($P < 0.05$) (Table 2). Low dose of GEN has no significant effect on the cisplatin induced decrease in cell viability.

Cell cycle analysis

Flow cytometry was conducted to analyse cell cycle by GEN. As shown in Table 3, granulosa cells exhibit higher level apoptosis, when they are exposed IC_{50} of cisplatin. Apoptosis was significantly decreased after addition of GEN ($P < 0.05$). Compared with control group, the percentage of cells at G0/G1 phase increased significantly in CDDP + GEN group and CDDP group. In addition, the percentage of CDDP group significantly decreased, compared with the control group at S and G2/M phase, while the percentage of CDDP + GEN group significantly increased compared with the CDPP group and close to control group at S and G2/M phase.

Table 1. Effect on granulosa cells of ovary by cisplatin at different concentration and time.

(μg/ml)	24h		48h		72h	
	Absorbency	Inhibtion ratio (%)	Absorbency	Inhibtion ratio (%)	Absorbency	Inhibtion ratio (%)
0	0.2827±0.0102		0.2914±0.0106		0.3003±0.0109	
0.25	0.2825±0.0111	0.11±0.02	0.2820±0.0122	0.19±0.01	0.2826±0.0121	0.12±0.02
0.5	0.2821±0.0108	0.20±0.03	0.281±0.0098	0.50±0.19	0.2818±0.0098	0.42±0.20
1	0.2765±0.0103*	4.67±1.63*	0.2618±0.0060	7.34±1.50	0.2712±0.0120*	5.76±1.16*
2.5	0.2443±0.0125*	14.34±1.82*	0.2300±0.0098	18.51±1.82	0.2387±0.0105*	16.32±1.54*
5	0.2012±0.0193*	26.56±3.83*	0.1888±0.0116	33.12±5.41	0.1953±0.0116	30.63±4.70
10	0.0753±0.0989*	78.92±6.19*	0.0332±0.1810	88.24±5.62	0.0569±0.1024*	84.30±7.77*

Different mark represent the significant difference at $p < 0.05$.

Table 2. Effect on granulosa cells of ovary by cisplatin combined with genistein at different concentration and time.

Group	Concentration		Inhibtion ratio (%)		
	CDDP (μg/mL)	GEN (mol/L)	12 h	24 h	48 h
CDDP	5	0	40.58±4.94	30.24±2.12	36.32±4.11
CDDP+GEN 1	5	5×10^{-6}	36.66±2.75	27.09±3.06	30.71±5.20
CDDP+GEN 2	5	1×10^{-6}	18.78±4.65*	15.80±2.51*	19.00±1.18*
CDDP+GEN 3	5	2×10^{-6}	15.25±2.86*	9.37±3.72*	9.69±3.22*
CDDP+GEN 4	5	4×10^{-6}	6.38±1.04*	5.42±1.71*	5.41±2.04*

Different mark represent the significant difference at $p < 0.05$.

Table 3. Effect on granulosa cells of ovary by cisplatin and cisplatin combined with genistein at different concentration and time,

Group	Cell cycle			
	G_0/G_1	S	G_2/M	Apoptosis
Control	61.41±2.35	26.80±1.52	20.13±1.10	2.547±1.05
CDDP	72.36±2.37*	15.47±1.14*	16.57±1.01*	6.46±1.18*
CDDP+GEN	67.31±2.47*△	26.01±2.02△	18.29±1.05	3.33±1.04△

Different mark represent the significant difference at $p < 0.05$.

DISCUSSION

Cisplatin (CDDP), an important anti-cancer drug, could cause serious damage to ovary tissue by harming the granulosa cells of ovary. This may cause ovarian deficiency and infertility (Dube et al., 1998). Previous study showed that toxicity was associated with plasma lipid peroxides (Previati et al., 2006). Moreover, other study have shown that CDDP combined with DNA caused cell damage (Olas et al., 2006; Salsbury et al., 2006), which could cause apoptosis to some extent. Our study showed that high dose of CDDP caused apoptosis of granulosa cells of ovary at S phase, which was consistent with previous reports that chemotherapeutics have significant role in interfering with cell cycle (Horowitz et al., 2004; Crescenzi et al., 2006; Xu et al., 2007; Hara et al., 2006).

Genistein has been shown to have many biological activities, such as anti-cancer, anti-oxidant, anti-inflammatory actions and inhibition of tyrosine-specific protein kinases (Akiyama et al., 1987; Rusin et al., 2010; Park et al., 2010; Zhang et al., 2008). GEN has become a popular candidate for drug development because of these features. In the present study, it was found that high dose of GEN could inhibit damage of granulosa cells of ovary caused by CDDP, which was consistent with previous study that GEN inhibit cell damage by chemo-therapeutics (Nynca et al., 2006), however, the detailed

molecular mechanisms of GEN inhibition cell damage was unclear so far, which would depend on further study.

Conclusion

The present study demonstrated that GEN could decreese cell damage of ovary induced by CDDP, which demonstrated that GEN is an effective drug to treat ovarian disorders induced by chemotherapeutics. The current study provides an effective approach for studying the mechanism underlying the pathogenesis of premature ovarian failure at cell level and treating premature ovarian failures. What is more, considering its low toxicity, GEN can be one of the potential drugs for more treatments of human diseases than now.

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Full Length Research Paper

Lentivirus-mediated shRNA targeting decoy receptor 3 (DcR3) inhibits proliferation and augments apoptosis in pancreatic cancer Capan-1 cells

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Pancreatic cancer is one of the most dismal malignancies with the actual 5-year survival of only 10 to 20%. Decoy receptor 3 (DcR3) is highly expressed in various cancer cells and plays a significant role in immune suppression and tumor progression. However, how DcR3 expression is modulated in pancreatic cancer cells is enigmatic. The aim of this study was to characterize the expression of DcR3 in pancreatic carcinoma and to evaluate the role of DcR3 in cell proliferation and apoptosis, which may lead to the development of novel treatments for this disease. In the present study, we examined five cell lines including three cell lines from pancreatic cancer (SW1990, Capcan-1 and PANC-1) and two cell lines from colon cancer (HCT116 and RKO) for DcR3 expression by reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative real time PCR. 3′-(4, 5 dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay and flow cytometric analysis were used to measure cell proliferation and apoptosis, respectively in the human pancreatic cancer Capan-1 cells. The human pancreatic cancer Capan-1 cell line was first infected with lentivirus-mediated shRNA and then analyzed by real-time PCR, and the results were further confirmed by Western blotting. DcR3 protein in our experimental results showed significant expression among the pancreatic cancer cell lines and their decreased expression by lentivirus-mediated shRNA resulted in the inhibition of cell proliferation and augmentation of apoptosis. In conclusion, these findings suggest that lentivirus-mediated gene therapy targeting DcR3 is a potential and attractive strategy for the treatment of pancreatic cancer.

Key words: Pancreatic cancer, decoy receptor 3 (DcR3), Capcan-1, shRNA.

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related deaths in United States (Jemal et al., 2009). Current treatment of pancreatic cancer includes surgery, chemotherapy, and radiotherapy, but these therapies are unsatisfactory, time consuming and expensive. Surgical abscission remains the only option for long term survival of patients.

Even after curative resection, the actual 5-year survival is only 10 to 20% (Awasthi et al., 2011; Yip-Schneider et al., 2005). Therefore, new options for treatments of pancreatic cancer are desperately needed. Gene therapy provides a new concept and it may become the new emerging direction for the development of treatment method for pancreatic cancer.

Decoy receptor 3 (DcR3), also known as TR6 or M68, is one of the members of tumor necrosis factor receptor (TNFR) superfamily. DcR3 mRNA (full-length 1.2 kb), its coding gene M68 located in human chromosome 20q13.3, processing mature the DcR3 271 amino acids, the molecular weight of about 35 KD. DcR3 cDNA encodes a

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300-aa protein containing the four tandem cysteine-rich repeats characteristic of the TNFR superfamily and lacking a transmembrane sequence (Pitti et al., 1998). DcR3 can bind with the Fas receptor, LIGHT and TLIA combination, regulate the process of tumor cell apoptosis. Thus, DcR3 is a potential target for treatment of cancer (Shen et al., 2005; Wu et al., 2003). Previous studies revealed that DcR3 is highly expressed in esophageal cancer, stomach cancer, colon tumors, but little is known about the expression of DcR3 in pancreatic cancer cells. Therefore, the purpose of this study was to characterize the expression of DcR3 in pancreatic carcinoma and to evaluate the role of DcR3 in cell proliferation and apoptosis in pancreatic cancer cells. In this study, we examined 5 cell lines including three cell lines from pancreatic cancer (SW1990, Capcan-1 and PANC-1) and two cell lines from colon cancer (HCT116 and RKO) for DcR3 expression by reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative real time PCR. Our results demonstrated that the DcR3 protein was significantly expressed among the pancreatic cancer cell lines and their decreased expression resulted in the inhibition of cell proliferation and augmentation of apoptosis.

MATERIALS AND METHODS

Chemicals and reagents

Cell culture medium reagents and 3′-(4, 5 dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), and dimethyl sulfoxide (DMSO) were purchased from Sigma. Fetal bovine serum (FBS) was purchased from the Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. China. Annexin V-FITC apoptosis detection kit was purchased from Beyotime Institute of Biotechnology Shanghai, China. Mouse anti-β-actin and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology. Ponceou and cell lysis buffer for Western and IP were purchased from Bio SS Beijing.

Cell culture

Human pancreatic adenocarcinoma cells SW1990 and Capan-1, human pancreatic epithelioid carcinoma PANC-1, human colon adenocarcinoma cells HCT116 and RKO were obtained from the American Type Culture Collection (Manassas, VA, USA), and cultured in the media recommended by the vendor (RPMI-1640 medium for SW1990 and Capan-1; Dulbecco's modified Eagle's medium for PANC-1 cells; Minimum essential medium Eagle for HCT116 cells; Leibovitz's L-15 medium for RKO cells) supplemented with 10% (v/v) FBS (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd ,China), 100 U/ml of penicillin and 100 µg/ml of streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were seeded in 10 cm culture dish and allowed to grow to approximately 70% confluence before experimentation.

Lentivirus transduction and transfection

Lenti-shRNA-DcR3 and lenti-NC virus plasmids were transfected in Capan-1 cells, as described previously (Fish and Kruithof, 2004). Capan-1 cells were transfected with ViraPower packaging mix using 100 µl lipofectamine 2000 reagents according to the manufacturer's instructions. The viral supernatant was harvested 48 h after transfection, passed through 0.45 µm filters and was allowed to concentrate, and the viral titer was determined. The viral supernatant was added into target lenti-1 cells at multiplicity of infection (10, 100, 200) with DcR3 and 5 µg/ml polybrene to obtain stably-transfected DcR3-1KD and DcR3-1NC cells.

Real-time RT-PCR

The isolated RNA subjected to RT-PCR was treated with DNase to avoid amplification of DNA contaminants. The forward and reverse primers are as follows: human DcR3 (GenBank Accession NM_003823.2/NM_032945.2), TCA ATG TGC CAG GCT CTT C and CTG GAA AGC CAC AAA GTC G; GAPDH (GenBank Accession NM_002046), ATT CCA CCC ATG GCA AAT TC and TGG GAT TTC CAT TGA TGA CAA G. Cycle threshold (C_t) method was used to analyze the results. The C_t value, which is inversely proportional to the initial template copy number, is the calculated cycle number in which the fluorescence signal emitted is significantly above background levels. The mRNA expression level of target genes was normalized to GAPDH using the $2^{-\Delta\Delta Ct}$ method, in which ΔC_t = target gene C_t – GAPDH C_t, and $\Delta\Delta C_t$ = ΔC_t treatment – ΔC_t control.

Western blotting

To confirm effect of lenti-shRNA-DcR3 on expression of DcR3 in Capan-1 cells, western blot analysis was performed as described previously (Rasul et al., 2012). Briefly, Capan-1 cells were incubated with lenti-shRNA-DcR3 or lenti-NC for indicated time. Cells were trypsinized, collected in 1.5 ml centrifuge tube and washed with PBS. The cell pellets were resuspended in lysis buffer and were lysed on ice for 30 min. After centrifugation for 15 min, the supernatant fluids were collected and the protein content of the supernatant was measured by the NanoDrop 1000 spectrophotometer (Thermo scientific, USA). The protein lysates were separated by electrophoresis on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ). The membranes were soaked in blocking buffer (5% skimmed milk) for 2 h. To probe for DcR3 and β-actin, membranes were incubated overnight at 4°C with relevant antibodies, followed by appropriate horseradish peroxidase (HRP) conjugated secondary antibodies and enhanced chemiluminescence (ECL) detection.

Cell proliferation assay

The cytotoxic effects of the shRNA on the cells were determined by MTT assay. Briefly, Capan-1 cells were seeded at a density of 1×10^4 cells per well in 96-well plates and were allowed to grow overnight. Upon exposure of the cells to lenti-shRNA-DcR3 or lenti-NC for different time periods, cell viability was measured by MTT assay. After incubation, growth of cells was determined by adding MTT (5 mg/ml in phosphate buffered saline) to each well and incubated for 4 h. After removal of the medium, dimethyl sulfoxide (DMSO) was added to each well and was shaken carefully. The absorbance was read at a wavelength of 490 nm in a plate reader (ELX 800, BIO-TEK Instruments Inc). The growth curve was plotted against mean values which were calculated using the following equation:

$I\% = [A_{490}\ (control) - A_{490}\ (treated)]/A_{490}\ (control) \times 100$

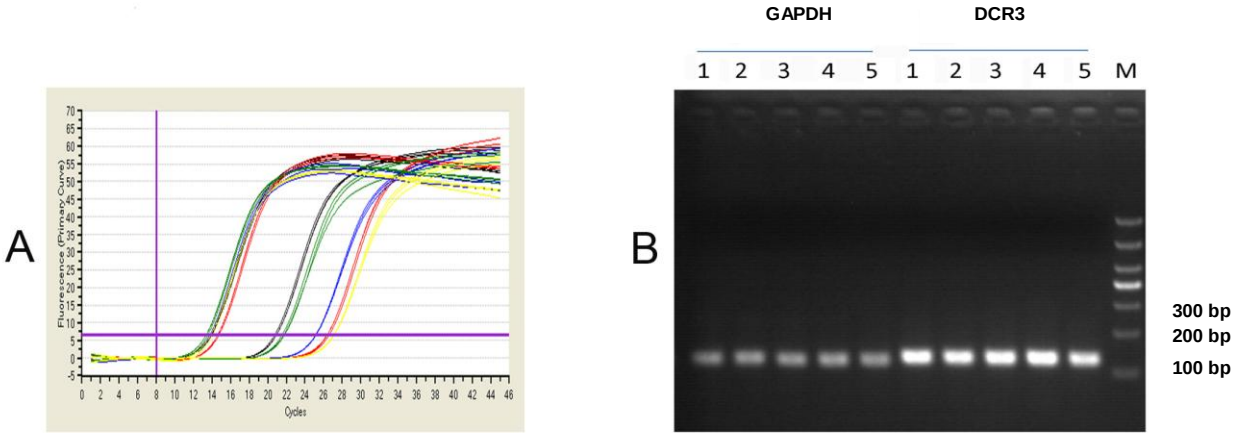

Figure 1. Real-time PCR and reverse transcriptase-PCR analysis of DcR3 expression in different cancer cells: (A) Real-time plot obtained for the quantification of the DcR3 expression in HCT116, PKO, SW1990, Capan-1 and PANC-1 cells (represented by black, red, blue, green, yellow curve respectively). HCT116 cells (black) line SW1990 (blue), in Capan-1 (green) amplification curve CT value, indicating that the curve of the DcR3 gene that corresponds to the initial template, its expression value was higher. (B) DcR3 and GAPDH mRNA expression levels of HCT116 (1), PKO (2), SW1990 (3), Capan-1 (4) and PANC-1(5) cells were assessed by standard RT-PCR. GAPDH served as a loading control.

Flow cytometric determination of apoptosis

The apoptotic rate of Capan-1 cells was examined by flow cytometry using annexin V-FITC/PI staining as described by us previously (Rasul et al., 2011; Shawi et al., 2011). Briefly, Capan-1 cells were cultured in 6-well plates and allowed to attach overnight. Cells were treated with lenti-shRNA-DcR3 or lenti-NC for different time periods. Cells were then collected, washed and resuspended in PBS. Apoptotic cell death was measured by double staining annexin V-FITC and PI using the Annexin V-FITC apoptosis detection kit (Beyotime Biotechnology Shanghai, China) according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after staining. Data acquisition and analysis were performed by flow cytometry (Beckman Coulter, Inc., Brea, CA) using Cell Quest software.

Statistical analysis of data

For the statistical analysis of data, comparisons between results from different groups were analyzed with SPSS for Window Version 15.0. Student's *t*-test was employed to determine the statistical significance of the difference between different experimental groups and control group. $P<0.05$ value was defined as statistically significant. All experiments were repeated at least three times. Data were presented as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

In this study, we used real time-PCR and reverse transcriptase-PCR to investigate the presence of DcR3 expression in five cell lines including three cell lines from pancreatic cancer (SW1990, Capcan-1 and PANC-1) and two cell lines from colon cancer (HCT116 and RKO). Human colon cancer cell line HCT116 and RKO were used as the positive control for DcR3 expression. Real-time plot obtained for the quantification of the DcR3 expression in HCT116, PKO, SW1990, Capan-1, PANC-1 cells, which is represented by black, red, blue, green, yellow curve, respectively for cDNA as a template DCR3 gene primers and GAPDH primers to amplify the target gene and reference gene. HCT116 cells (black), SW1990 (blue), and Capan-1 (green) amplification curve threshold cycle (CT) value lower, indicating that the curve of the DcR3 gene that corresponds to the initial template, its expression values were higher (Figure 1A). Furthermore, DcR3 and GAPDH mRNA expression levels of HCT116 (1), PKO (2), SW1990 (3), Capan-1 (4), and PANC-1(5) cells were assessed by standard RT-PCR. For reverse transcriptase-PCR analysis of DcR3 expression in different cancer cells, results showed that among three, two human pancreatic adenocarcinoma cell lines (SW1990 and Capan-1) have high levels of DcR3 protein, while slightly less level was observed in case of PANC-1 human pancreatic carcinoma cell line (Figure 1B). A previous study (Elnemr et al., 2001) also reported high levels of DcR3 expression in Capan-1 cells. Thus, keeping in view those findings, we selected the Capan-1 cells for the subsequent experiments.

DcR3, also known as TR6 or M68, is one of the members of TNFR superfamily. DcR3 plays a major role in allowing cancer cells to evade attack by the immune system (Elnemr et al., 2001; Li et al., 2007; Pitti et al., 1998). Additional studies have demonstrated that there is a significant correlation between the overexpression of DcR3 and resistance to FasL-mediated apoptosis in cancer cells (Elnemr et al., 2001; Pitti et al., 1998; Shen et al., 2005). Thus, DcR3 is a potential target in treatment of cancer (Shen et al., 2005; Wu et al., 2003). In this study we have constructed five different shRNAs targeting the DcR3 expression in Capan-1 cells.

RNA interference (RNAi) is a powerful tool for studying protein function (Wang et al., 2007; Zhang et al., 2008).

The effective delivery of siRNA molecules into target cells or tissues is critical for successful RNAi application. Lentiviral vectors can effectively transduce exogenous genes with the long-term expression of transgenes (Fish and Kruithof, 2004) and have a promising future in clinical applications (Thomas et al., 2006; Wang et al., 2007). To determine the role of DcR3 in Capan-1 pancreatic cancer cells, we constructed lentiviral vectors for the delivery of short hairpin RNA, a precursor, into Capan-1 pancreatic cancer cells to suppress the DcR3 gene expression. As shown in Figure 2A to D, the number represents (1) negative control group (ddH2O), (2) positive control group (without shRNA fragment with empty vector), (3) Marker (5, 3, 2, 1.5 and 1 kb, 750, 500, 250, and 100 bp), (4 to 8) (A) PSC-1, 2,3,4,5, (B) PSC-1, 2,3,4,5, (C) PSC-1, 2,3,4,5, and (D) PSC-1, 2,3,4,5. These results suggest that there was no exogenous nucleic acid contamination, which was confirmed by lane number-1 (ddH_2O) in the system, while lane number-2 is positive group, which confirmed that lane 2 (positive group) for the empty vector clone shRNA (306bp band) is not connected to the target gene. The lane numbers 4 to 8 are positive results confirming for the different RNA interference the target shRNA fragment (that is PSC1/PSC2/positive clones) and virus vector cloning PSC3/PSC4 (PCR fragment size was 343 bp) in the lane 4 to 8 were selected for the sequencing, and sequencing results confirmed that the target gene (PSC1/PSC2/PSC3/PSC4,) was accurately inserted into the viral vector.

As shown in Figure 2E, (1) negative control group (ddH_2O), (2) negative control group (empty eukaryotic expression vector), (3) positive control (GAPDH), (4) Marker, and (5 to 12) TNFRSF6B (1 to 8 transformants). The findings of the present study demonstrated that lane number 1 (negative control group) confirmed that the system has no exogenous nucleic acid contamination, while lane 2 (positive group) for the empty vector (310 bp band) is not connected to the target gene. The lane 3 confirmed connection as a positive control for the GAPDH reference gene carrier. The lanes number 5 to 12 indicated the clear bar with a confirmed carrier is connected to the target gene (the size of the PCR primers for the 1125 bp bands) below the fuzzy bands not connected to the empty vector into the target gene bands. The lanes number 5 to 12 in the PCR positive clones were sent for sequencing and the sequencing results fully consistent with the goal of sequencing, which confirmed that the target gene is accurately connected to the eukaryotic expression vector.

Western blot analysis of three different groups including control group (CON) is not transfected with any plasmid in Capan-1 cells (empty cells in control group), NC was transfected with expression plasmid and negative control cell group of viral vector plasmid (negative control group). KD1, 2, 3, 4 contain the target genes for the different interference target sequences of RNAi viral vector plasmid serial number. The corresponding interference plasmid serial were KD1 (4209), KD2 (4210), KD3 (4211), and KD4 (4212). Control group (CON group) and NC group have DcR3 protein expression, indicating that the empty virus vector plasmid DcR3 gene does not significantly inhibited the DcR3 gene expression. KD groups (KD1, KD2 and KD4 groups) significantly decreased DcR3 protein expression as compared to CON and NC group, while KD3 group has no significant influence protein expression. It was noted that out of 4209, 4210, and 4212 targets, 4210 is the most effective shRNA to knockdown the expression of target gene (DcR3) (Figure 2F).

The human pancreatic cancer Capan-1 cell line was infected with shRNA-DcR3 and then analyzed by real-time PCR, and the results were confirmed by Western blotting. Therefore, in the present study, five RNA interference lentiviral vectors were constructed to down regulate the expression of DcR3 in Capan-1 pancreatic cancer cells. Our results demonstrated that these shRNA downregulated the DcR3 expression in Capan-1 cells, then we selected shRNA, which significantly decreased the levels of DcR3 in human pancreatic capan-1 cells (Figure 2A to E). The results of the present study were further confirmed by western blot analysis, which demonstrated that shRNA decreased the DcR3 expression in pancreatic cancer cells (Figure 2F).

Morphological changes were observed under microscopy after treating cells with lentivirus-mediated shRNA targeting DCR-3 resulting in the decreased number of cells as compared to the control group and cells became rounded and shrinked, which were polygonal in untreated cells (Figure 3A). Furthermore, our immunofluorescence results demonstrate the successful virus infection of target cells and the infection rate was more than 80% (Figure 3A). In addition, to analyze the effect of lentivirus-mediated shRNA targeting DcR-3, equal amounts of concentrated media from cells were subjected for reverse transcriptase-PCR with the DcR3 primer. Our results illustrate the percentage of relative amount of mRNA in different groups. It was found that Capan-1 cell sampled cDNA as a template to amplify the target gene and reference gene by using DcR3 gene primers. The KD group significantly reduced DcR3 gene expression in comparison to NC group (Figure 3B). To further confirm these findings, Capan-1 cells were exposed to lentivirus-mediated shRNA targeting DcR-3 for specified time intervals. Equal amounts of lysate protein were subjected to gel electrophoresis. Expression levels of DcR-3 were monitored in control group (CON), NC, and KD target knockdown in Capan-1 cells by Western blot assay. Our results reveal that DcR3 protein expression was significantly downregulated in KD group as compared to the control group (Figure 3C). These results are in consistent with our previous results.

To determine whether lentivirus-mediated shRNA targeting DcR-3 inhibits pancreatic cancer Capan-1 cell proliferation or not, therefore, we examined its effects on

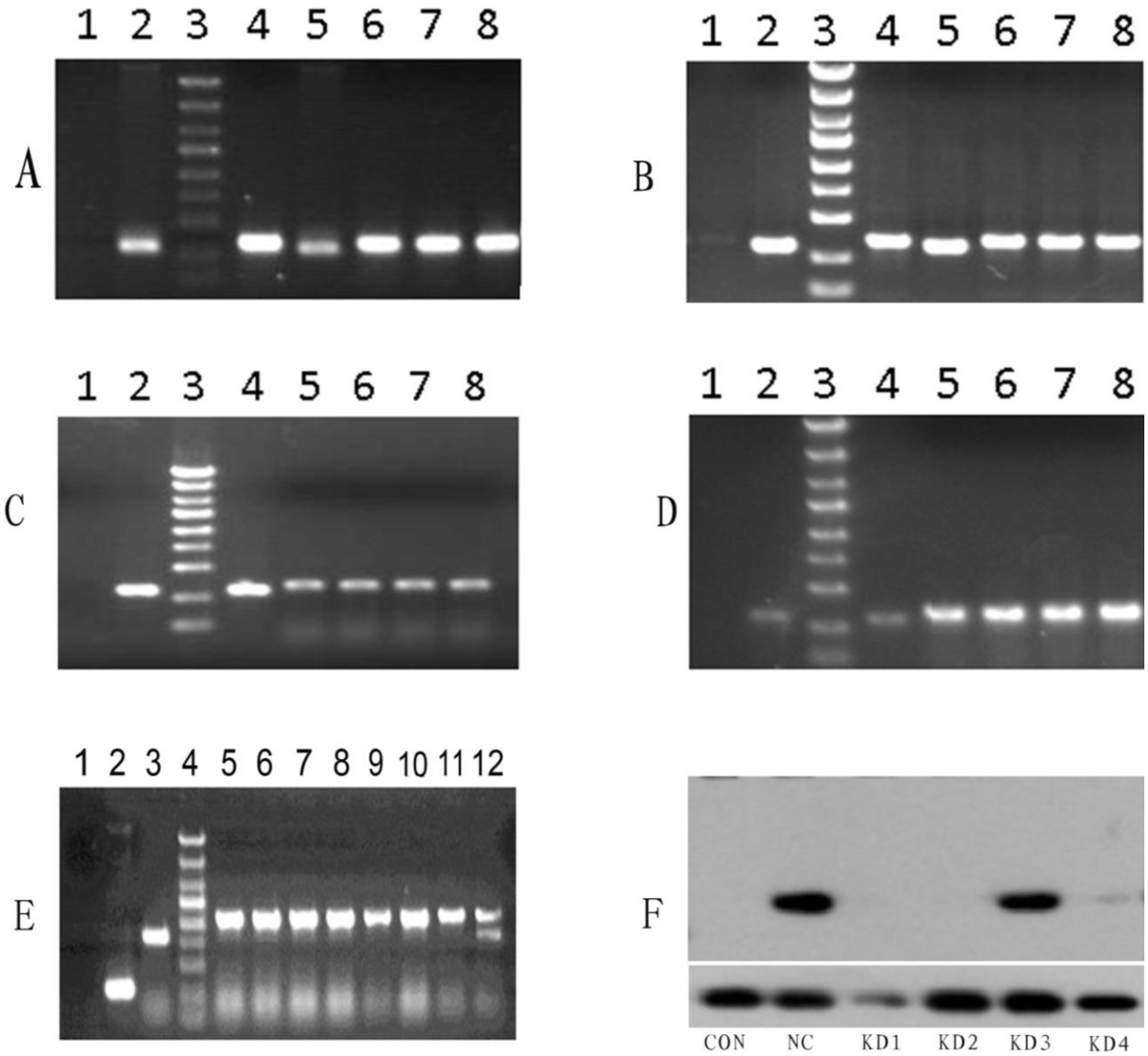

Figure 2. Reverse transcriptase-PCR and immunoblotting assessment of various shRNAs on decoy receptor 3 (DcR3) mRNA and protein expression in Capan-1 pancreatic cancer cells: (A-D) The number represents (1) negative control group (ddH2O), (2) positive control group (without shRNA fragment with empty vector), (3) marker (5, 3, 2, 1.5 and 1 kb and 750, 500, 250 and 100 bp), (4-8) (A) PSC-1, 2,3,4,5; (B) PSC-1, 2,3,4,5; (C) PSC-1, 2,3,4,5; (D) PSC-1, 2,3,4,5; (E) (1) negative control group (ddH2O), (2) negative control group (empty eukaryotic expression vector), (3) positive control (GAPDH), (4) Marker, and (5-12) TNFRSF6B (1-8 transformants). (F) Western blot analysis of three different groups including control group (CON), which is not transfected with any plasmid in Capan-1 cells (empty cells in control group), NC was transfected with expression plasmid and negative control cell group was transfected with viral vector plasmid (negative control group). KD1, 2, 3, 4 contain the target genes for the different interference target sequences of RNAi viral vector plasmid serial number. The corresponding interference plasmid serial were KD1 (4209), KD2 (4210), KD3 (4211), and KD4 (4212). The data shown are representative of three independent experiments with the similar results.

the growth of pancreatic cancer Capan-1 cells by quantifying the viable cells after treating cells with lentivirus-mediated shRNA targeting DCR-3 for various time periods using MTT assay. The results reveal that lentivirus-mediated shRNA targeting DCR-3 inhibited the growth of pancreatic cancer Capan-1 cells in a time-dependent manner (Figure 4A).

Apoptosis, autophagy, and necrosis are the major types of cell death (Leist and Jaattela, 2001). Among the three major pathways of cell death, apoptosis is the most well planned and orderly mode of cell death (Elmore, 2007; Hengartner, 2000). More than 50% of neoplasms undergo aberrations in the apoptotic machinery which leads to abnormal cell proliferation (Mashima and Tsuruo, 2005;

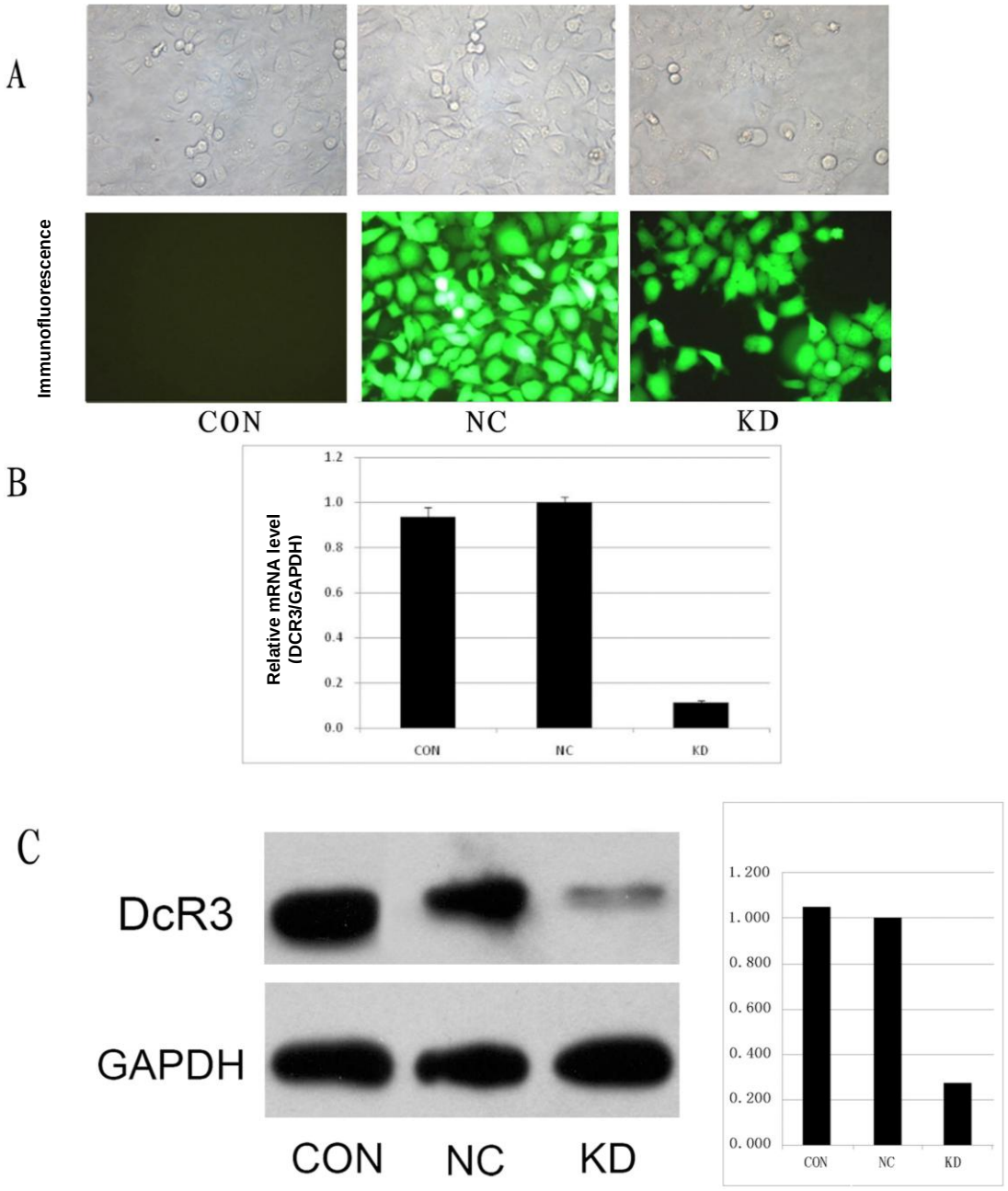

Figure 3. Effects of lentivirus-mediated shRNA targeting DcR-3 on cell morphology and reverse transcriptase-PCR and immunoblotting assessment of decoy receptor 3 (DcR3) mRNA and protein expression in Capan-1 pancreatic cancer cell line: (A) Morphological changes and fluorescence images (200 ×g, 200 magnification bright field image) of Capan-1 pancreatic cancer cells after the transfection with lentivirus-mediated shRNA (KD) as compared to the control group (CON). (B) Equal amounts of concentrated media from cells were subjected for reverse transcriptase-PCR with the DcR3 primer, representative graph illustrating the percentage of relative amount of mRNA in different groups. (C) Capan-1 cells were exposed to lentivirus-mediated shRNA targeting DcR-3 for specified time intervals. Equal amounts of lysate protein were subjected to gel electrophoresis. Expression levels of DcR-3 were monitored in the control group (CON), NC, and KD (knockdown) groups in Capan-1 cells by Western blot assay. Data are representative of at least two independent experiments with similar results.

Pommier et al., 2004). The regulation of apoptosis is therefore the most important in the treatment of cancer (Fulda, 2010; Lawen, 2003; Reed, 2002). Accumulated evidences indicated that most of the chemotherapeutic agents halt tumor cells proliferation via induction of apoptosis (Saha et al., 2010; Wu et al., 2009; Zhang,

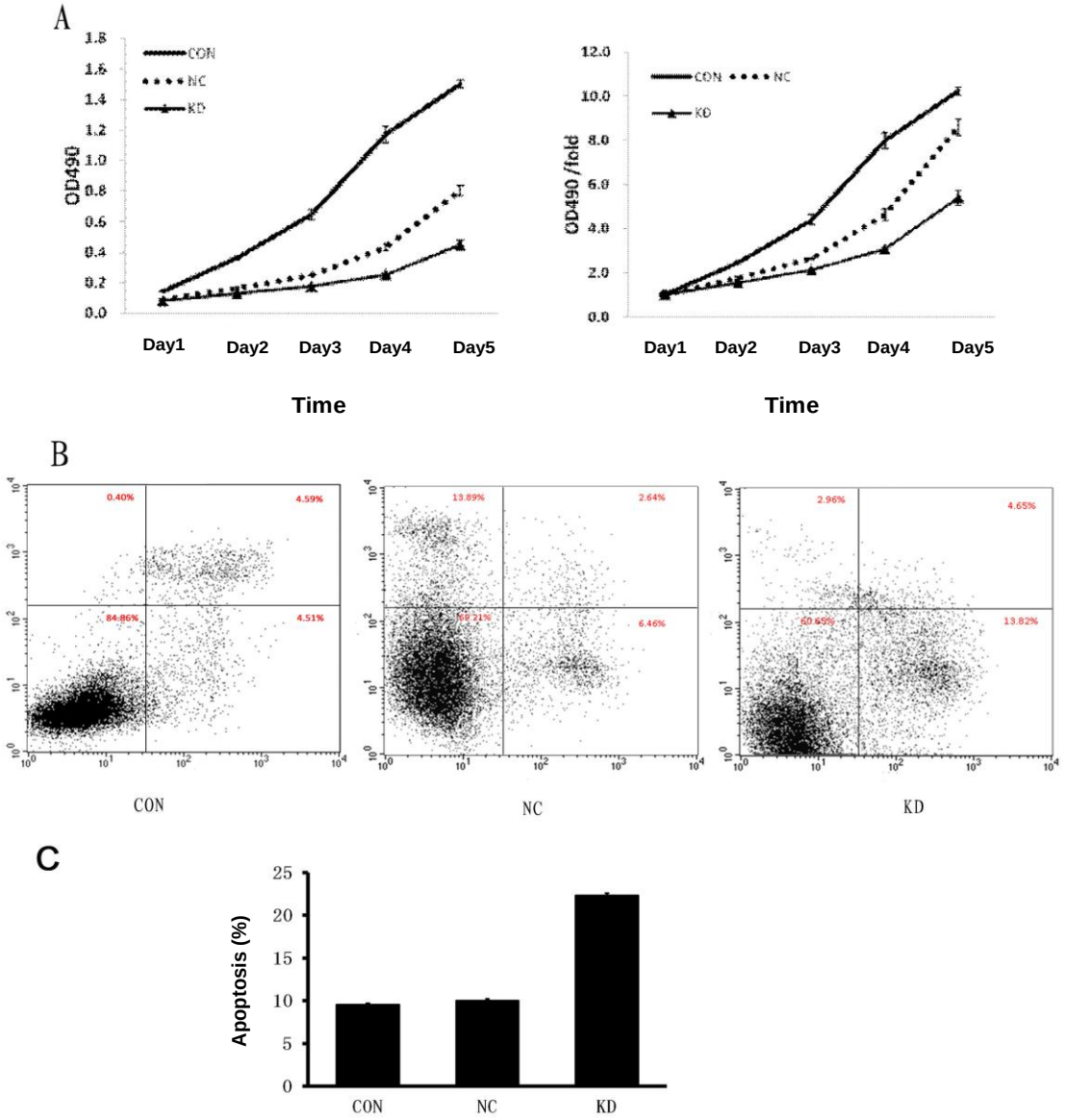

Figure 4. Lentivirus-mediated shRNA targeting DcR-3 inhibits pancreatic cancer Capan-1 cell proliferation and induces apoptosis: (A) Cell viability was determined by MTT assay. The effect of lentivirus-mediated shRNA on the cell growth inhibition of Capan-1 cells was compared with control group. The graph was plotted against mean values of percentages of three independent experiments. (B) Apoptosis induced by lentivirus-mediated shRNA targeting DCR-3 in pancreatic cancer Capan-1 cells. Capan-1 cells were treated with lentivirus-mediated shRNA. Then cells were stained with FITC-conjugated Annexin V and PI for flow cytometric analysis. The flow cytometry profile represents Annexin V-FITC staining in *x* axis and PI in *y* axis. The number represents the percentages of apoptotic cells in each condition. (C) Representative graph illustrating the percentage of apoptotic cells. Statistically significant changes were compared with control group. The data shown are representative of three independent experiments with the similar results.

2002). Here in, we examined whether lentivirus-mediated shRNA targeting DCR-3 inhibited cell growth of pancreatic cancer Capan-1 cells through the induction of apoptosis. Lentivirus-mediated shRNA-induced apoptosis was determined by flow cytometric analysis. The results of flow cytometric analysis showed that the rates of apoptosis were 28.87 and 8.43% in the cells treated with lenti-sh-RNA-DcR3 or lenti-NC, respectively as compared to the 7.31% in control cells (Figure 4B and C). Our results demonstrated that shRNA decreased DcR3 expression and enhances apoptosis in human pancreatic cancer Capan-1 cells.

Taken together, our results suggest that the shRNA-mediated decrease in the levels of DcR3 significantly inhibited cell proliferation and enhanced apoptosis in human pancreatic cancer cells. These results suggest that lentivirus-mediated gene therapy targeting DcR3 is a potential and attractive strategy for the treatment of pancreatic cancer.

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