AN ABSTRACT OF DISSERTATION OF

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Title: Effects of Medicinal Herbs on Contraction Rate of Cultured Cardiomyocyte. Possible Mechanisms Involved in the Chronotropic Effects of Hawthorn and Berberine in Neonatal Murine Cardiomyocyte.

Abstract approved

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    Herbs have been used for many centuries in diverse civilizations for the treatment of heart disease. Only a few natural supplements claim to have direct cardiovascular actions including hawthorn (*Crataegus spp.*) and berberine derived from the *Berberidaceae* family. Several different studies indicate important cardiovascular effects of hawthorn and berberine. For example, both exert positive inotropic effects and have been used in the treatment of congestive heart failure.

    Recently, it was shown that hawthorn extract preparations cause negative chronotropic effects in a cultured neonatal murine cardiomyocyte assay independent of beta-adrenergic receptor blockade. The aim of this study was to further characterize the effect of hawthorn extract to decrease the contraction rate of cultured cardiomyocytes.

We hypothesized that hawthorn extract may be acting through muscarinic receptors to decrease contraction rate of cardiomyocytes. Atrial and ventricular cardiomyocytes were treated with hawthorn extract in the presence of atropine or himbacine. Changes in the contraction rate of cultured cardiomyocytes revealed that both muscarinic antagonists significantly attenuated the negative chronotropic activity of hawthorn extract. Using quinuclidinyl benzilate, L-[benzylic-4,4'-$^3$H] ([$^3$H]-QNB) as a radioligand antagonist, the effect of a partially purified hawthorn extract fraction to inhibit muscarinic receptor binding was quantified. Hawthorn extract fraction 3 dose-dependently inhibited [$^3$H]-QNB binding to mouse heart membranes. These findings suggest that muscarinic receptors may be involved in the negative chronotropic effect of hawthorn extracts in neonatal murine cardiomyocytes.

Berberine exhibits variable positive and negative chronotropic effects in different species. Our first aim was to examine the effect of berberine in a cultured neonatal murine cardiomyocyte assay. Our study demonstrates that berberine has significant negative chronotropic actions on cardiomyocytes which is not an effect of beta-adrenergic receptor blockade.

Pertussis toxin (PTX), a Gi/o protein inhibitor, blocked the negative chronotropic activity of berberine. Muscarinic, adenosine, opioid, and $\alpha_2$ receptors are coupled through a G-protein (Gi/o) to adenylyl cyclase in an inhibitory fashion. Activation of these receptors are primarily responsible for PTX-sensitive negative chronotropic effects in heart. We hypothesized that berberine may be acting through one of these receptor type to decrease contraction rate of cardiomyocytes. For this purpose, we studied the effects of the muscarinic-receptor antagonists, atropine, himbacine, or AF-

DX 116 on the negative chronotropic activity of berberine. Muscarinic antagonists completely blocked the effect of berberine on contraction rate of cardiomyocytes, whereas the bradycardic effect of berberine was not inhibited by the opioid, adenosine, or $\alpha_2$ receptor antagonists naloxone, CGS 15943, or phentolamine, respectively.

Using [$^3$H]QNB as a radioligand, we demonstrated that berberine bound to muscarinic receptors of adult mouse heart membranes with relatively high affinity. Furthermore, berberine dose-dependently inhibited [$^3$H]QNB binding to muscarinic M2 receptors exogenously expressed in HEK 293 cells. Therefore, the findings of the present study suggest that berberine has muscarinic agonist effects in cultured neonatal murine cardiomyocytes, potentially explaining reported physiological effects of berberine.

Cardiac hypertrophy represents the most important factor in the development of congestive heart failure. We investigated the inhibitory effect of berberine on hypertrophy of H9c2 cells. In rat heart-derived H9c2 myoblast cells treated with different hypertrophic agonists such as insulin growth factor II (IGF-II), arginine vasopressin (AVP), phenylephrine, and isoproterenol, protein content and size of cells were significantly increased compared to control group. However, the number of H9c2 cells after treatment with hypertrophic agonists did not differ significantly compared to control. The increases in area of cells and protein content induced by the hypertrophic agonists were inhibited by treatment with berberine in a concentration-dependent manner. Our findings have provided the first scientific evidence that

berberine may have an inhibitory effect on hypertrophy of heart-derived cells, and provide a rationale for further studies to evaluate berberine's cardiac activity.

Effects of Medicinal Herbs on Contraction Rate of Cultured Cardiomyocyte.
Possible Mechanisms Involved in the Chronotropic Effects of Hawthorn and
Berberine in Neonatal Murine Cardiomyocyte.


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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Chapter 1:  Introduction

Hawthorn

Significance of research on herbal medicines such as hawthorn

Estimates for the year 2006 are that 80 million people in the United States have one or more forms of cardiovascular disease (CVD), including high blood pressure, 73 million; coronary heart disease,16 million; myocardial infarction (acute heart attack), about 8 million; angina pectoris (chest pain or discomfort caused by reduced blood supply to the heart muscle), about 10 million; stroke, about 7 million; and Heart Failure, about 6 million (http://www.americanheart.org). Many additional Americans have significant risk factors for infarction, including hypertension, hypercholesterolemia, diabetes, tobacco use, and depression.

In many nations, herbal therapies are among the most popular of all 'alternative' treatments. In the United State, use of herbal medicine increased 380% from 1990 to 1997. Herbs have been used for many centuries in different conteries for the treatment of heart disease. They use different natural products and antioxidant vitamins to treat or prevent the hypercholesterolemia, coronary heart disease, and congestive heart failure (CHF) (Gavagan, 2002).

Herbal therapies have shown promise in terms of efficacy. Recently, the National Institutes of Health (NIH) acknowledged the importance of herbaltherapies and developed a strategy regarding research in this area for cardiovascular conditions. Primary health physicians should be aware of cardiovascular indications, adverse effects and drug interactions of herbal treatments (Ernst, 2003; Gavagan,  2002).

 Only a few natural supplements, such as hawthorn, claim to have direct cardiovascular effects. Hawthorn is widely used in Europe and Asia (Hobbs et al., 1990). Several studies (in vivo and in vitro) have shown that hawthorn extract has

different pharmacological actionsin the cardiovascular system including increasing coronary blood flow, improving myocardial contractility, decreasing blood pressure, and increasing cardiac performance (Daniele et al., 2006). Thus, hawthorn extracts (e.g. Heart Care® tablets) are sometimes used to self-medicate for hypertension or CHF.

Patients with congestive heart failure (CHF) experience limitations on their breathing, mobility and life expectancy. CHF patients commonly use inotropic agents such as phosphodiesterase type 3 (PDE3) inhibitors and cardiac glycosides; but Arrhythmia, can be a significant side effect limiting the usefulness of PDE3 inhibitors and glycosides medications in heart failure treatment. On the other hand, hawthorn extracts apparently possess vasodilatory and cardiotonic effects and also appears to be antiarrhythmic and capable of improving rhythmicity in cardiomyocytes. The potential usefulness of hawthorn in the therapy of arrythmias and heart failure is suggested by multiple studies of the cardiovascular actions of hawthorn (Tauchert et al., 2002; Pittler et al., 2003; Long et al., 2006).

It is necessary and important that research on the cardiovascular effects of hawthorn to be continued. Some of the studies analysed individual components of hawthorn extracts to separate and identify its active compounds. The beneficial effects of an herbal product such as hawthorn may be come from different compounds which acting synergistically; therefore their adverse effects and interaction should be addressed.

Variation of hawthorn

There are few plant groups as confusing to botanists as hawthorn. Hawthorn (*Crataegus*) is a genus in the rose (Rosaceae) family and is known by the common names English hawthorn, haw, maybush, and whitethorn. There are now about 280 species recognized in northern temperate regions, such as North America, Europe, and Asia. Sixty or more species are known from Europe and Asia. In eastern North America, one of the most extensively planted hawthorn species is the English hawthorn, *Crataegus laevigata* (Hobbs et al., 1990; Rigelsky and Sweet, 2002). *Crataegus laevigata* (Poir) and *Crataegus monogyna* Jacq are most commonly used in Western medicine (Daniele et al., 2006). It is a bright red fruit-bearing plant with bright green three- to five-lobed leaves and white flowers (Rigelsky and Sweet, 2002). The generic name *Crataegus* is derived from a Greek word *Kratus*, or strength, referring to the hardness of the wood (Daniele et al., 2006).

The constituents of hawthorn (Plant and chemical constituents)

Hawthorn fruits, leaves and flowers are considered the most potent parts of the plant. They contain a number of chemical components which possess some active properties in animals, such as flavonoids (quercetin, quercitrin, catechin, rutin and oligomeric proanthocyanidin), triterpene saponins (oleanolic acid, ursolic acid and crataegus acid) and amines (β-phenethylamine, tyramine, and acetylcholine) (Rajendran 1996, Zhang 2001; Chang, 2002). However, no single compound has yet been isolated to be responsible for hawthorn's cardiotonic effects. Flavonoids, particularly oligomeric proanthocyanidins of *Crataegus* extract are major bioactive

constituents that possess potent antioxidant activity. The flavonoids are divided into different subclasses including flavanols (catechin and proanthocyanidin), flavanones (naringin and hesperidin), isoflavanones, flavonols (quercetin and myric Etin), flavones (luteolin and apigenin) and anthocyanidins (Manach et al, 2004; Chang et al., 2005; Bahorun et al, 1994).

Timing of harvest, method of extraction (water, ethanol, methanol), and different parts of plant are important factors to consider when developing hawthorn drugs For example, procyanidins is found in the fall leaves almost three times of the spring leaves (Hobbs et al., 1990; Yao et al., 2008).

Today, commercial preparations, manufactured in Europe, are standardized to contain flavonoids, oligomeric procyanidins, and chlorogenic acid, among other constituents. The daily dose tested in clinical trials is 160-1800 mg of standardized extract containing 2.2% flavonoids or 18.75% oligomeric proanthocyanidins (Daniele et al., 2006). It is worth to discuss evidence for each of the various reported effects of hawthorn on the cardiovascular system.

## Anti-hyperlipidemic effect of hawthorn

The lipid-lowering property of the hawthorn extract has been shown in animal studies. There are still few well-designed human clinical trials to support this effect, eventhough a decrease of serum lipid levels was reported in some heart failure studies. Therefore, future placebo-controlled, randomized studies are needed to investigate the efficacy of hawthorn to substantiate usefullness in the treatment of hyperlipidemia (Chang et al., 2005).

To date, hawthorn is shown to decrease average cholesterol levels and triglyceride levels in the serum of hyperlipidaemic volunteers. Hawthorn also reduced serum levels of lipid peroxidate malonic diadehyde indicating a strong antioxidative effect (Chen, 1995).

Hawthorn's ability to decrease body fat and blood lipids was studied on rats. It was reported that hawthorn significantly decreased body fat, total cholesterol levels and triglyceride level in rats compared with control. On the other hand, the high density lipoprotein (HDL) level was greater in the hawthorn group (Chen et al., 1995). In another study, the extract of hawthorn reduced low density lipoprotein (LDL) cholesterol in rats. Hawthorn extract was also shown to increase bile acid excretion and to depress hepatic cholesterol synthesis in these rats (Rajendran et al., 1996).

An alcoholic extract of the berries of hawthorn, *Crataegus oxyacanyha,* was found to cause a very significant decrease in the total cholesterol levels of serum and cardiac tissue of rats. The authors suggest that it may be due to the activity of the extract in increasing hepatic 7α- hydroxylase activity (Akila, 2008). It is reported that an alcoholic extract of the berries of hawthorn significantly increased in cholesterol 7α-hydroxylase activity (Zhang et al., 2002).

Hawthorn extract protects LDL cholesterol from copper-II and peroxyl radical induced oxidation (Quettier-Deleu et al., 2003). Since some flavonoids such as quercetin have been reported to block the oxidative modification of LDL cholesterol and thus prevent associated cytotoxicity (de Whalley et al., 1990), therefore hawthorn

extract rich in flavonoids may have protective effects via this mechanism (Chang et al., 2005).

The mechanisms by which hawthorn lowers LDL cholesterol are not fully understood, but one possibility is the up-regulation of the hepatic LDL receptors reported by Shanthi et al, 1996, which may help in reducing plasma LDL/VLDL levels. Rajendran et al (1996) have reported that an alcoholic extract of the berries of hawthorn, *Crataegus oxyacantha*, significantly increased the binding of LDL cholesterol to hepatocyte membranes and promoted the influx of plasma cholesterol into the liver, suggesting an increase in hepatic LDL-receptor activity (Rajendran, 1996).

Supplementation with hawthorn dry fruit powder lowers the serum levels of total cholesterol and triglyceride in rabbits fed with 12 weeks of a high cholesterol diet (Zhang et al, 2002). It was reported that supplementation of hawthorn extract blocked the intestinal acyl CoA:cholesterol 7α- hydroxylase (ACAT) activity instead of affecting the hepatic-3-hydroxy-3-methyl glutaryl CoA (HMG-CoA) reductase activity in rabbits fed with a high cholesterol diet (Zhang et al., 2002). Since the intestinal ACAT plays a role in cholesterol absorption and the hepatic HMG-CoA reductase pathway is involved for cholesterol biosynthesis, this indicates that hawthorn may block cholesterol absorption rather than cholesterol synthesis (Chang et al., 2005). Therefore, the anti-hyperlipidemic activity of hawthorn may be mediate via different mechanisms which a more complete understanding of their actions on a cellular level is warranted and necessary.

Chronotropic and inotropic cardiac effects of hawthorn

It is reported that hawthorn extract may induce positive inotropic effects in rat heart (Petkov et al. 1981) and in patients with CHF (Schwinger et al., 2000). The mechanism(s) underlying the positive inotropic effect of hawthorn remains unclear. It was suggested that an increase of the intracellular calcium concentration and inhibition of the cAMP phosphodiesterase activity may play a role in the hawthorn effect (Petkov, 1981). On the other hand, Muller et al. (1999) found that hawthorn extract LI132 had no influence on the L-type calcium current in guinea pig ventricular cardiomyocyte, indicating that the positive inotropic effect of hawthorn was probably not caused by cAMP phosphodiestrase inhibition (Muller et al. 1999). Additionally, hydroalcoholic extract WS 1442[®] of hawthorn increased the contractile force of isolated papillary muscle strips via a cAMP-independent pathway (Schwinger et al. 2000; Chang et al., 2005). Moreover, Hawthorn extract LI 132 elicits a significant positive inotropic effect on the contraction amplitude of rat cardiomyocyte. The inotropic effect of hawthorn may be attributed to an increased $Ca^{2+}$ sensitivity, i.e., increased $Ca^{2+}$ affinity of troponin and/or altered crossbridge kinetics as cytosolic $Ca^{2+}$ did not appear to be altered (Popping et al., 1995). However, the mechanisms underlying the positive inotropic effect of hawthorn remain unclear.

Hawthorn has been observed to show various effects on heart rate in different studies. It was reported that hawthorn caused a negative chronotrophic effect in vivo and in vitro such as neonatal murine cardiomyocyte (Long et al., 2006; Regelsky and Sweet, 2002), whereas the isolated guinea pig heart increased in heart rate (Schussler

et al., 1995a; Popping et al., 1995). In Langendorff preparations of isolated perfused rabbit hearts, heart rate was increased by vitexin-rhamnoside and vitexin and decreased by hyperoside. In spontaneously-beating Langendroff-guinea pig hearts, monodactyl-vitexinrhmnoside from hawthorn concentration-dependently enhanced heart rate up to 17% (Schussler et al., 1995b). It was shown that hawthorn extract of leaves and stems or dried berries caused a reduction in contraction rate of cultured murine cardiomyocytes. On the other hand, fractionation of hawthorn extract produced multiple, separable chronotropic activities, including fractions with positive chronotropy and fractions with negative (Long et al, 2006).

The reason for these interspecies differences is not known. Attempts to define an active chronotropic component in hawthorn extract have not identified a single factor yet. Some groups point to the flavonoids of hawthorn as the source of direct cardiac activity.


## Vasodilatory effect of hawthorn

Hawthorn extract (*Crataegus* fruit) showed a concentration-dependent relaxation in rat isolated mesenteric arteries; this effect decreased when the endothelium was denuded (Chen et al., 1998). The effect of hawthorn extract was similar to that of acetylcholine, the endothelium-dependent vasodilator. The relaxation effect of hawthorn extract was also inhibited by a nitric oxide (NO) synthase inhibitor, $N_w$-nitro-L-arginine methyl ester (L-NAME). Similarly, L-NAME blocked the acetylcholine-induced relaxation in the same arteries (Chen et al. 1998), indicating that the endothelium-derived relaxation factor NO may be involved in hawthorn-

induced vasodilatation (Chang et al., 2005). *Crataegus* extract caused endothelium-dependent vasodilatation by stimulation of NO production and cGMP accumulation in isolated aortic preparation of the rat (Kim, 2000). Therefore, it is suggested that a lowering of arterial blood pressure and enhancement of coronary blood flow may be mediated by hawthorn extract (Veveris et al., 2004; Cheng et al., 2005).

Another study showed that monoacetyl-vitexinrhamnoside (AVR), a flavonoid from *Crataegus* with phosphodiesterase (PDE)-inhibitory properties, reduced developed tension in rabbit isolated femoral artery rings. Vasodilation by AVR decreased after inhibiting EDRF formation. Thus, the vasodilatory action of AVR may be mediated by EDRF in addition to PDE-inhibition (Schussler et al. 1995). Whether AVR is sufficient to mediate hawthorn's vasodilatory effects is not known.

The activation of $Ca^{2+}$-activated $K^+$ channels but not ATP-sensitive $K^+$ channels may play a minor role in the relaxant action of hawthorn extract. Hawthorn extract may have a direct inhibitory effect on $Ca^{2+}$ entry through voltage-sensitive $Ca^{2+}$ channels (Chen et al. 1998). These studies (in vivo and in vitro) suggested the ability of hawthorn extracts to affect ion channels and stimulation of NO production to cause vasodilation and increased coronary blood flow.

## Coronary blood flow and hawthorn

It was reported that hawthorn extract increases coronary blood flow in the isolated heart as well as in whole animal models (Upton et al. 1999). This effect may be mediated by an enhanced endothelial synthesis of NO (Veveris et al. 2004).

The flavonoid O-glycosides luteolin-7-glucoside, hyperoside, and rutin increased coronary flow (186%, 66%, and 66% respectively) dose-dependently with maximum values being obtained at a concentration of 0.5 mmol/l in Langendorff perfused isolated guinea pig hearts. Similarly, the C-glycosides vitexin, vitexin-rhamnoside, and monoacetyl-vitexin-rhamnoside caused a slight coronary vasodilation (20%, 7%, and 10% respectively) (Schussler et al. 1995a).

In Langendorff preparations of isolated perfused rabbit heart, vitexin-rhamnoside and vitexin increased coronary flow while hyperoside resulted in a decrease of coronary blood flow (Occhiuto et al. 1986).

### Antioxidant effects of hawthorn

Oxidants play an important role in a wide variety of normal biological reactions, but overloading of these species ($H_2O_2$, $O_2^-$, HOCl), especially those released by inflammatory cells, may be involved in the pathogenesis of many diseases. These oxidants have also been implicated in myocardial ischemic injury (Bahorun et al., 1996). They may damage endothelial cells and monocytes via lipidic peroxidation and oxidation of protein sulfhydryl groups, leading to disturbances in membrane permeability and enzyme function. *Crataegus monogyna* extracts might attenuate some oxidative stress and tissue damage caused by $H_2O_2$, $O_2^-$ and HOCl, major reactive oxygen species produced by activated phagocytic cells. The antioxidant activity of *Crataegus monogyna* extract may be bound to the total phenolic proanthocyanidin and flavonoid contents (Bahorun et al., 1996).

The extracts of hawthorn plants subjected to drought and cold stress (environmental stress treatments) not only give higher of polyphenolics including epicatechin and hyperoside but also possess higher antioxidant capacity, as compared with control nonstressed plants (greenhouse-grown plants). It is indicated that these kinds of stress treatments can increase the secondary metabolites and total of antioxidant capacities in leaves of hawthorn (Kirakosyan et al., 2003).

Hawthorn fruit extracts containing (-)-epicatechin, chlorogenic acid, hyperoside, isoquercetin, protocatechuic acid, rutin, and quercetin were found to be effective in preventing the peroxyl radical-induced oxidation of α-tocopherol human low-density lipoprotein (LDL) (Zhang et al., 2001; Bahorun et al., 2003). In fact, it has been suggested that protective effects of different hawthorn extracts in various cardiovascular disorders (hyperlipidemia, ischemia/reperfusion injury, anti-arrhythmetia, hypertrophy and CHF) may be related to its antioxidant activity (Zhang et al., 2001; Chang et al., 2005; Hwang and Bleske, 2008).

Anti-arrhythmic effect of hawthorn

Hawthorn appears to exhibit some cardioprotective effects due to anti-arrhythmic properties and it improves rhythmicity in neonatal murine cardiomyocytes (Chang et al., 2005; Long et al., 2006). It was reported that several species of *Crataegus* may prevent cardiac arrhythmias induced by adrenaline, aconitine, and calcium chloride (Ammon and Handel, 1981). Hawthorn extract WS 1442[®] dose-dependently blocked ventricular tachycardia following ligation of the left coronary artery (Veveris et al. 2004). However, it was mentioned that long-term application of hawthorn

preparations might be more effective. For example, hawthorn extract may prevent reperfusion arrythmias after 7 min of coronary occlusion after short-term (7 days) pretreatment (Krzeminski et al., 1993), but its effects seemed to be better in the chronic (3 months) treatment group (Al Makdessi et al., 1999). In the rat myocardial ischemia model, hawthorn extract significantly decreased the incidence and severity of ischemia-related arrhythmia (Garjani et al., 2000).

The mechanism(s) underlying the anti-arrhythmic effect of hawthorn preparations remains unclear. It was reported that Crataegus extract prolongs the referactory period and increases the action potential duration in isolated guinea pig ventricular cardiomyocyte by blocking repolarizing potassium currents (Muller et al., 1996 and 1999), an effect similar to the class III anti-arrhythmic drug. This might be the possible mechanism of anti-arrhythemic effects of hawthorn extract. On the other hand, concerns have been raised regarding the roles of free radicals in the induction of reperfusion arrhythmia (Gaudal and Duvellerrory, 1984). Its active component flavonoids showing strong antioxidant activity, these may be responsible for anti-arrhythmic effects of hawthorn (Chang et al., 2005).

### Hawthorn and hypertension

Human studies investigating the hypotensive effects of hawthorn are limited. Walker et al (2002) have mentioned that hawthorn extract did not significantly decrease systolic and diastolic pressure in a small group of patients with mild hypertension.

Nevertheless, it was reported that hawthorn (up to 1200 mg extract/day for 16 weeks) caused a significant drop in diastolic pressure in patients with diabetes taking medication (Walker et al., 2006).

Kocyildiz et al., (2006) have suggested that hyperoside from hawthorn extract caused hypotension resulting from increased NOS and NO levels. Further human clinical trials are still needed to verify the anti-hypertensive effects of hawthorn preparations (Chang et al., 2005).

## Congestive heart failure and hawthorn

It was reported that *Crataegus* Special Extract WS 1442 (CSE) does not reduce heart failure progression in patients who have heart failure. Hawthorn extract appears to increase the risk of HF progression (Zick et al., 2008). However, hawthorn may improve symptoms in patients with CHF even if disease progression continues.

The clinical effects of hawthorn extract on patients with congestive heart failure were evaluated over two decades ago. Most of the studies used specialized hawthorn extract such as WS 1442[®] or LI 132 for standardization of its active components (Schroder 2003). The dosage, frequency, duration of treatment and the percentages of "active" components in exact preparations varied, although attemps are made to standardize some preparation to percent flavonoids. A meta-analysis study to assess the beneficial effects of hawthorn extracts analyzed results from eight well-designed clinical trials (Pittler et al., 2003).  The clinical parameters most often employed included subjective symptoms, exercise capacity or tolerance, blood pressure, heart rate and rate-pressure products. The physiological outcomes of maximal workload

and heart rate-blood pressure product both showed significant improvement in patients treated with hawthorn extracts. Exercise tolerance also had a marginal non-significant increase (Baughman and Bradley, 2003).

Hwang et al., (2009) have shown that hawthorn (*Crataegus oxycantha* extract WS 1442[®]) treatment caused modest beneficial effects on cardiac remodeling and function during long-term, pressure overload-induced heart failure in a rat model of aortic constriction (Hwang et al., 2009).

Clinical studies of hawthorn are complicated by the presence of other drugs. Cardiac glycosides, beta-blockers, diuretics and angiotensin-converting-enzyme inhibitors (ACE-I) are standard treatment for congestive heart failure, and hawthorn is usually evaluated as an adjunctive therapy (Schroder et al., 2003). This leads to difficulties interpreting true benefits of hawthorn. NIH/NCCAM funded hawthorn studies have been content to simply look at safety of hawthorn in drug combinations rather than effects.

One of the mechanisms attributed to heart failure progression is a decrease in the activity of endothelial nitric oxide synthase (eNOS). It was reported that hawthorn extract has potential effects on nitric oxide by improving endothelium-dependent relaxation in rat atrial vessels. Catechin, epicatechin, and quercetin increased eNOS activity. On the other hand, increased activity of inducible nitric oxide synthase (iNOS) is thought to contribute to many aspects of myocardial dysfunction and maladaptive myocardial dysfunction. Hawthorn has been shown to decrease the expression of iNOS. In addition, it was mentioned that quercetin and epicatechin from

hawthorn caused a decrease in iNOS expression (Kim et al., 2004; Garcia-Mediavilla et al., 2007; Hwang et al., 2008).

Recently, the immunomodulatory response to heart failure has become a major focus of research. Immunomodulatory mediators (IL-1ß, IL-2, IL-6, IL-8, and TNF-α) have been found to be elevated in heart failure and considered to be important in the development and progression of heart failure. It was reported that hawthorn did not affect the immunomodulatory response following long term aortic constriction in a rat model of heart failure. Therefore, the hawthorn's effect in treating HF (if any) may not be immunomodulatory based (Bleske et al., 2007).

## Hawthorn and myocardial infarction

Myocardial infarction is the primary cause of mortality in developed and developing countries. Myocardial infarction usually results from sudden decrease in coronary blood flow to a segment of the myocardium, which initiates a continuum of progressively more severe cellular changes that finally causes cell death and tissue necrosis (Dargie, 2005)

Hawthorn extract was shown to exhibit cardioprotective effects in in vitro and in vivo models of ischemia/reperfusion (Li, 1984; Jayalakshmia, 2004).

Veveris et al., (2004) reported that the area of myocardial infarction within the ischemic zone was significantly smaller in rat treated with *Crataegus* special extract WS1442[®] compared with controls (Veveris et al. 2004).

An alcoholic extract isolated from the fruits of hawthorn shows protective effect in the rat model of isoproterenol-induced myocardial infarction. Hawthorn also

prevented the isoproterenol-induced decrease in antioxidant enzyme activity (e.g. glutathione peroxidase, superoxide dismytase and catalase) (Jayalakshmi et al., 2004). Therefore, it has been suggested that the protective effects of hawthorn extract in ischemia/reperfusion injury may be related to its antioxidant activity (Chang et al., 2005).

The flavonoids in alcoholic extract of *Crataegus oxycantha* probably protect the heart from myocardial damage by scavenging free radicals and thereby suppressing the peroxidation of lipids (Jayalakshmi et al. 2006). Superoxide dismutase (SOD) is a class of enzymes which catalyzes the dismutation of two superoxide radicals to form hydrogen peroxide and molecular oxygen. Overexpression of mitochondrial SOD at a targeted intracellular location protects the heart against ischemic injury. Alcoholic extract of *Crataegus oxycantha* treatment protected the mitochondria against isoproterenol-induced depletion of SOD. Alcoholic extract of *Crataegus oxycantha* pretreatment maintained mitochondrial antioxidant status, prevented mitochondrial lipid peroxideative damage and decrease in Kreb's cycle enzymes induced by isoproterenol in rat heart (Jayalakshmi et al. 2006).

The clinical studies to show the efficacy of hawthorn extract in patients with ischemic heart diseases is limited (Veveris et al., 2004). The therapeutic effects of *Crataegus* extract in patients with angina pectoris showed favorable results (Hanak and Bruckel, 1983; Weng et al., 1984), but the numbers of patients were not sufficient. Since coronary heart disease is one of the leading public health problems in the modern society, it should be valuable to evaluate the anti-ischemic effects of the hawthorn extract in a well-designed prospective clinical trial (Chang et al., 2005).

Hawthorn and cardiac hypertrophy

Hawthorn treatment counteracts myocardial dysfunction in early overload-induced cardiac hypertrophy in rats. It modifies left ventricular remodeling in a hypertrophied group and causes a greater degree of compensatory hypertrophy compared with vehicle treatment (Hwang and Bleske, 2008). It was suggested that hawthorn may prevent or impede the transition to a maladaptive eccentric or dilated hypertrophy and heart failure. The hypertrophy effects of hawthorn require more study. One mechanism that may be responsible for the transition from hypertrophy to a maladaptive form is the generation of reactive oxygen species (ROS). Therefore, there is a possibility that hawthorn may influence cardiac remodeling during pressure overload by decreasing the generation of ROS (Hwang and Bleske, 2008).

Many studies have reported that atrial natriuretic factor (ANF) mRNA levels increase in response to various hypertrophic stimuli such as β-adrenergic stimulation, hypertension, and coronary artery occlusion. Hwang et al., (2009) showed that hawthorn (*Crataegus oxycantha* special extract WS 1442®) significantly blocked the increase in ANF mRNA induced by 5 months of pressure overload. These reports suggest that hawthorn extract may be involved in ventricular remodeling. Further human studies are necessary to determine the effect of hawthorn extract on ventricular remodeling (Hwang et al., 2009).

Adverse effects of hawthorn

Herbal medicines are popular but health care professionals often feel uncertain about their risks and adverse effects or potential harm. Large populations of patients

try herbal treatments; therefore it seems relevant for health care professionals and cardiologists to be aware of cardiovascular adverse effects of herbal products (Ernst, 2003).

The adverse effects caused with different preparations of hawthorn within the most commonly applied dose ranges are usually mild and tolerable. The hawthorn extracts most used in the clinical trials were WS 1442® (extract of hawthorn standardised to 18.75% oligomeric procyanidins) and LI 132 (extract of hawthorn standardised to 2.25% flavonoids). Most of hawthorn's adverse effects were rash, sweating, headache, dizziness and vertigo, palpitation, agitation, sleepiness and gasterointestinal symptoms. Larger doses in animal studies were reported to cause sedation, hypotension and arrhythmia. In New York Heart Association (NYHA) functional class III heart failure, patients taking doses up to 1800 mg per day showed only mild adverse events such as dizziness and vertigo, which is statistically no different from those with 900 mg per day, and even those with placebo (Tauchert, 2002; Daniele et al., 2006). The adverse reactions associated with hawthorn extract (900 mg per day) are usually mild and infrequent (Schroder 1990; Tauchert 1999). Therefore, it is suggested that hawthorn extract is safe and tolerable therapy for patients with cardiovascular diseases (Chang et al., 2005).


Drug interactions and hawthorn

The drug interaction profile of hawthorn extract has not been well studied. It may interact with some prescription medications or related cardioactive herbs. For example, hawthorn may increase vasodilation if it is used with vasodilating drugs

such as, caffeine and adenosine (Daniele et al., 2006; Rigelsky and Sweet, 2002; Upton and Petrone, 1999; Hahen et al., 1960).

In an NIH/NCCAM funded study, coadministration of hawthorn extract with digoxin caused only modest changes in digoxin pharmacokinetics which were not statistically significant. It was suggested that these differences are a result of mild P-glycoprotein induction likely due to the presence of quercetin and various quercetin glycosides in the hawthorn extract. In addition, there was no evidence of any pharmacodynamic interaction, as measured by electrocardiogram (ECG), heart rate, and blood pressure. In total, it was suggested that both drugs may be given together safely (Tankanow et al., 2003).

## Pharmacokinetic studies of hawthorn

Although pharmacologic effects of hawthorn have been attributed to flavonoids in general, and specific flavonoid present in hawthorn has been studied in isolation, the bioavailability of many hawthorn flavonoids argues against their cardioactivity in the whole extract.

Hawthorn extract or one of four constituents phenolic compounds (epicatechin, hyperoside, isoquercetin, chlorogenic acid) were given to male rats by oral gavage or intravenous dosing (Chang et al, 2005); only epicatechin was absorbed unchanged.

Liquid chromatography-coupled mass spectrometry was developed for in vivo pharmacokinetic studies of vitexin rhamnoside in rat plasma. Pharmacokinetics and bioavailability of vitexin rhamnoside were assessed in rats after intravenous and oral administration. The oral bioavailability of vitexin rhamnoside was only 3.57%, which

suggested that vitexin rhamnoside was poorly absorbed or underwent extensive first-pass metabolism (Liang et al., 2007). It was shown that there were no significant differences in the absorption of vitexin-2-O-rhamnoside in different segments of rat intestine such as duodenum, jejunum, ileum, and colon. However, coadministration of vitexin-2-O-rhamnoside with P-glycoprotein (P-gp) inhibitors increased the absorption of vitexin-2-O-rhamnoside in rats (Xu et al., 2008).

After oral administration of quercetin to rats, only 6.7% was absorbed unchanged, and the remainder was transformed in the gut wall to quercetin glucuronide (Zue et al., 2006). Approximately half (55.8%) is bioavailable. Therefore, absorption of quercetin depends on formation of quercetin glucuronide by enzymes such as uridine diphosphate-glucuronosyltransferases in intestinal walls (Chen et al., 2005).

Quercetin is water-soluble and its absorption in the human small intestine is expected be poor. Absorption of quercetin following a meal containing quercetin-rich onion and tea was about 24% in ileostomy patients (Hollman et al., 1995). In human plasma, 99% quercetin is bound to plasma proteins, and only 0.3-1.8% unbound fraction in the plasma (David and Kristina, 1998). The half-life of quercetin is about 25 h in humans following oral administration (Hollman et al., 1995).

Pharmacokinetic studies are necessary to determine the absorption of leaf hawthorn extract (standardized to 12.9 mg/ml of oligomeric proanthocyanidins) from the small intestine in healthy adults in order to clear its safety (Yao et al., 2008).

Extraction procedure of hawthorn

Vierling et al. (2003) have reported that with an exhaustive extraction procedure using water-methanol or water-ethanol mixtures (40 to 70% v/v ethanol or methanol), extracts can be obtained which show no significant differences in their effects on relaxation of vascular smooth muscles that had been previously contracted by noradrenaline. However, an aqueous extract (100% water) showed a lower effect compared to the water-ethanol or water-methanol extracts. Examining the content of the extracts, it was noted that with the aqueous extract the content of procyanidins (8% instead of 13%), flavonoids (0.7% instead of 3.4%), and total phenols (14% instead of 25%) was substantially lower. Thus, the reduced effect of the aqueous extract was a quantitative and not a qualitative difference and must be administred at a higher dose.

The use of extraction solvents with similar polarity (40 to 70% v/v ethanol or methanol) and using the same experimental procedure, gave extracts which showed very similar spectra of constituents. It seems possible to produce bioequivalent extracts with comparable effect profiles by using 40 to 70% v/v ethanol or methanol as the extraction solvent (Vierling et al., 2003).

Berberine

Berberine is an isoquinoline derivative alkaloid isolated from many medicinal herbs, such as *Hydrastis Canadensis* (goldenseal), *Cortex phellodendri* (Huangbai) and *Rhizoma coptidis* (Huanglian), *Berberis aquifolium* (Oregon grape), *Berberis aristata* (tree turmeric), and *berberis vulgaris* (berbery) (Figure 1). Several in vivo and in vitro studies have shown that berberine, the major component of these herbs, has many potential pharmacological effects including inhibition of adipocyte differentiation, anti-cancer effects, anti-microbial effects, LDL-lowering effects and anti-inflammatory effects (Choi et al., 2006; Kim et al., 2008). It is also reported that berberine has antibacterial, local anesthetic and anticoagulant properties. Berberine has been shown to be effective in the treatment of acute diarrhea, and to antagonize the secretary effect of cholera toxin in isolated ileum and also animals (Bova et al., 1992).
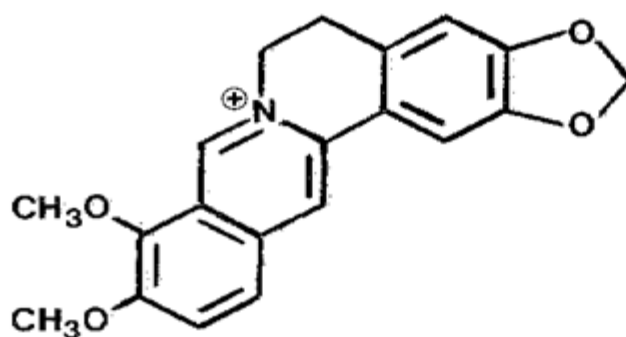


Figure 1. Chemical structure of berberine.

Both clinical trials and animal studies have suggested some beneficial effects of berberine, coptis and its extracts on the cardiovascular system. Berberine increased contractile force, decreased peripheral vascular resistance and lowered blood pressure (Lau et al., 2001; Shen, 1997). In China, some Chinese physicians use coptis or berberine in the treatment of cardiac diseases, including high blood pressure and congestive heart failure (CHF) (Zhao et al., 1994; Zhang, 1997; Hong et al., 2002).

## Chronotropic effect of berberine

Berberine showed negative chronotropic (heart rate) activity on cardiac pacemaker cells. In spontaneously beating right atria of guinea pig and sinoatrial cells of rabbit, berberine produced concentration-dependent negative chronotropic effects. The decrease in contraction rate was accompanied by a depression of the phase 4 depolarization without significant changes in other parameters of the nodal action potential. In atria of guinea pig exposed to atropine ($10^{-7}$ M), the bradycardiac activity of berberine was not altered (Shaffer, 1985; Riccioppo Neto, 1993) suggesting that muscarinic receptors are not involved. On the other hand, berberine did not alter the positive chronotropic activity of isoproterenol; suggesting that β-receptors are not involved (Riccioppo Neto, 1993). The mechanism underlying the negative chronotropic effect of berberine, however, remains elusive (Riccioppo Neto, 1993).

## Inotropic activity of berberine

It was reported that berberine had a strong positive inotropic (change in contractile length) activity in dogs, humans and isolated preparations from rabbits,

guinea pigs and rats (Sabir et al., 1978; Vik-Mo et al., 1983; Maroko and Ruzyllo, 1983). Shaffer, in his experiments, showed that berberine had a unique profile, producing negative chronotropic activity and positive inotropic effects in guinea pig isolated atria (Shaffer, 1985). The positive inotropic effect was apparently not mediated by stimulation of β- or α-adrenoceptors since this was unaffected by propranolol or phentolamine. The positive inotropic effect appeared to be caused by increasing the force-velocity relationship of cardiac myocytes (Shaffer, 1985). The positive inotropic effect of berberine may not due to inhibition of $Na^+$-$K^+$-ATPase, since berberine (100 µM) had no effect on the activity of guinea pig brain $Na^+$-$K^+$-ATPase (Cohen et al., 1978, Lau et al., 2001).

The electrophysiological action of berberine and its derivatives on cardiac myocytes is a lengthening of the action potential without affecting other action potential characteristics. This effect was reported in different species including guinea pigs, cats, rats, rabbits, dogs and humans. Selective lengthing of action potential duration would increase $Ca^{2+}$ influx through the $Ca^{2+}$ channels and elevate release of $Ca^{2+}$ from the sarcoplasmic reticulum which is required for enhanced cardiac contractility (Chi et al., 1996, Riccioppo Neto, 1993; Lau et al., 2001). Therefore, berberine has a potential in the treatment of CHF, if further human studies could demonstrate its efficacy and safety.

## Antiarrhythmic effect of berberine

It was reported that berberine suppressed arrhythmias induced in different species such as rats and dogs. An increase in the effective refractory period of purkinje fibers

may be the possible mechanism of antiarrhythmetic action of berberine. In vitro studies in mammals suggest that berberine exerts class III antiarrhythmic effects in cardiac muscle. The class III antiarrhythmic drugs act by blocking cardiac delayed rectifier $K^+$ channels (Lau et al., 2001).

In a clinical study, it was shown that berberine might be useful in a long-term therapy of ventricular premature beats. Frequency and complexity of ventricular premature beats were decreased and the left ventricular ejection fraction was increased by berberine after 2 weeks of treatment (Zeng and Zeng., 1999).

Delayed afterdepolarization is one of the important electrophysiological elements for cardiac arrhythmia. Berberine suppressed the amplitude of delayed afterdepolarization induced by ouabain in guinea pig and rabbit ventricular cells. Inhibition of $Na^+$ influx may be the possible underlying mechanism for the reduced amplitude of delayed afterpolarization by berberine. It is considered that a transient inward current carried primarily by $Na^+$, which is intimately associated with an increase in intracellular $Ca^{2+}$ overload, is responsible for the delayed afterpolarization. This effect is also likely to be involved in the antiarrhythmic action of berberine (Wang et al., 1994; Lau et al., 2001).

In isolated guinea pig papillary muscles, berberine (3-100 µM) blocked the shortening of action potential duration induced by an ATP-sensitive $K^+$ ($K_{ATP}$) channel activator. Patch-clamp data showed that 3-100 µM berberine inhibited $K_{ATP}$ channel activity. Cardiac ATP-sensitive $K^+$ ($K_{ATP}$) channels could serve as another potential target for the antiarrhythmic activity of berberine (Nakaya et al., 1991). The beneficial effect of berberine on ischemia-induced arrhythmias might be attributed to

inhibition of $K_{ATP}$ channel activation and subsequent shortening of action potential duration during ischemia (Lau et al., 2001). The anti-arrhythmic effect of berberine may be attributed to involvement of different channels such as $K^+$ and $Na^+$ channels.

Hypotensive effect of berberine

It was reported that intravenously administrated berberine transiently caused hypotension in dogs and other mammals. Intravenous injection of berberine decreased the mean arterial pressure in anesthesized rats (Kang et al., 2003). The hypotensive action of berberine has been attributed to its ability to block the release of $Ca^{2+}$ from internal stores. Protoberberine, a berberine derivative, lowered systolic blood pressure in hypertensive rats, in a dose-dependent manner (Liu et al., 1999). The mechanism of hypotensive action of protoberberine was attributed to a central sympatholytic effect (Lau et al, 2001).

Several mechanisms have been proposed for the hypotensive action of berberine. It was reported that berberine decreased blood pressure in rabbits by blocking α-adrenoceptors. Berberine was also reported to possess an antagonistic effect on α-adrenoceptor-mediated contraction of rat or rabbit aorta (Olmez and Ilhan, 1992; Lau et al., 2001). Additionally, Schmeller and co-workers have demonstrated that in porcine brain, berberine clearly binds to α-receptors, $\alpha_1$ and $\alpha_2$, with $IC_{50}$ of 3.2 and 0.4 µM, respectively (Schemller et al., 1997). On the other hand, berberine caused hypotension in dogs through a direct vascular action independent of adrenergic, cholinergic or histaminergic mechanisms (Sabir et al., 1978).

In rat isolated mesenteric arteries, berberine induced both endothelium-dependent and endothelium-independent relaxation. Ko and coworkers (2000) reported that NO plays a role in endothelium-dependent vasorelaxation and stimulation of $K^+$ channels by berberine. Blocking of $Ca^{2+}$ release is involved in endothelium-independent vasorelaxation. It was shown that berberine has an additional hypotensive effect, at least in part, via inhibition of angiotensin-converting enzyme (ACE) and increasing the production of NO in the vascular tissues (Kang et al., 2003). In general, the vasodilatory and hypotensive effects of berberine may be attributed to multiple cellular mechanisms like production of NO, antagonism of α-receptors, inhibition of ACE, decrease of $Ca^{2+}_i$, and stimulation of $K^+$ channels.

## Cardiac hypertrophy

Cardiac hypertrophy is the heart's response to different extrinsic (such as arterial hypertension or valvular heart disease) and intrinsic (familial hypertrophic cardiomyopathy) stimuli that impose increased biomechanical stress (Frey and Olson, 2003).

Cardiac hypertrophy can roughly be divided into physiological and pathological hypertrophy (Hunter & Chien, 1999). In shorter stresses, physiological hypertrophy is an adaptive response to maintain heart function by increasing the size of cardiomyocytes for powerful contraction. In contrast, prolonged hypertrophic stresses may cause non-compensatory pathological hypertrophy that will induce the fetal gene expression of atrial natriuretic factor (ANF) and brain natriuretic factor (BNF), and increase the risk of heart failure.Hypertrophy in response to pathologic signaling has

been considered an adaptive response to maintain cardiac output. On the other hand, prolonged hypertrophy is associated with a significant increase in the risk for sudden death or promoting of heart failure (Frey and Olson, 2003; Heineke & Molkentin., 2006; Chu et al., 2008).

However, the transition from physiological hypertrophy to pathological hypertrophy in terms of molecular mechanisms is unclear. Several extracellular signaling molecules such as insulin-like growth factors (IGFs), angiotensin II (ANG II), chronic norepinephrine and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) have been reported to be involved in the development of myocardial hypertrophy (Frey  and Olson, 2003; Heineke & Molkentin, 2006).

A hallmark feature in the progression of heart failure is cardiac ventricular remodeling which is attributed to hypertrophy and subsequent apoptosis of individual cardiomyocytes followed by fibrosis. This process is initiated by a variety of stimuli, including mechanical wall stress, paracrine and endocrine factors such as catecholamines, angiotensin-II, enothelin-1, growth factors and inflammatory cytokines (Johns et al., 2004; Frey and Olsen, 2003). As cardiac myocytes are terminally differentiated, hypertrophy is by an increase in myocyte size and growth of non-myocyte components. The defining features of hypertrophied cardiomyocytes are an increase in the rate of protein synthesis and a higher organization of the sarcomere. All these structural changes result in an increase in myocardial volume and mass; ultimately myocardial death results in impaired normal cardiac output (Hong et al., 2002).

It has been shown that the binding of IGF-I and IGF-II to the IGF1R induces cell hypertrophy in neonatal ventricular cardiomyocytes (Adachi et al. 1994, Miyashita et al. 2001). Several investigations further observed that activation of the IGF1R signaling cascade can improve heart contractions and attenuate pathological hypertrophy and fibrosis. However, the increased expression of IGF-II in several animal models with pathological cardiac hypertrophy raised doubts about the role of IGF-II in reducing stressful responses in the heart (Kluge et al. 1995, Lee et al. 2006).

It was suggested that by modulating Gαq and the activation of its downstream effectors PKC and CaMKII, the IGF2R signaling pathway may contribute to the progression of pathological hypertrophy. Chu et al., (2008) suggested that it may be possible that IGF2R signaling (by activating PKC-α and CaMKII) has a critical role in the regulation of pathological cardiac remodeling and the progression from adaptive cardiac hypertrophy to cardiac failure (Chu et al., 2008). Chu et al (2008) found that treatment with both IGF-I and IGF-II induced cell hypertrophy, but only IGF-II induced the protein level increase of ANP and BNP, which are the markers of pathological hypertrophy. They demonstrated that there are two signaling pathways, ERK1/2 and PKC-α/CaMKII, involved in IGF-II-induced cell hypertrophy, and that only PKC-α/CaMKII activation is needed for IGF-II-increased expression of ANP and BNP. Several studies have suggested that the activation of PKC-α and CaMKII signaling in response to calcium influx (Molkentin, 2006; Ferrero et al. 2007) might be attributed to the Gαq-induced pathological hypertrophy and apoptosis of cardiomyocytes (Braz et al. 2004).

A variety of extracellular factors (such as angiotensin II, norepinephrine, endothelin-1 (ET-1), insulin-like growth factor (IGF)-I, interlerkin-1ß) act at their transmembrane receptors to trigger a series of intracellular signalling events which cause hypertrophy (Hong et al, 2002). Catecholamines such as noradrenaline (NA) act as neurotransmitters of sympathetic nerves. NA will increase left ventricular weight-to-body weight ratios and up-regulate atrial natriuretic factor (ANF) gene expression to promote hypertrophy.  NA induces myocardial hypertrophy in both primary cell cultures and in animal model (Morgan and Baker, 1991; Bhambi et al., 1991; Hong et al., 2003). NA is also believed to be involved in the process of hypertensive myocardial hypertrophy. It was suggested that there is a positive relationship between the levels of noradrenaline and adrenaline and mortality of patients with congestive heart failure (Swedberg et al., 1990; Hong et al., 2003). Activation of adrenoceptors could be an effector in both promoting and maintaining myocardial hypertrophy from increased cardiac sympathetic nerve activity and elevated levels of circulating catecholamines (Hong et al, 2003).

Circulating arginine vasopressin (AVP) causes hypertrophic effects via V1 receptors. V1 receptors act through a Gq coupled signaling mechanism to stimulate phospholipase C to produce inositol trisphosphate and 1,2-diacylglycerol, which respectively increase the release of $Ca^{2+}$ from internal stores and then activate protein kinase C (PKC). It was reported that vasopressin increases intracellular $Ca^{2+}$ $[Ca^{2+}]i$, followed by activation of MAP kinases and the release of atrial natriuretic factor in isolated cardiomyocytes (Van der Bent et al., 1994; Brostrom et al, 2000).

**Hypertrophy signaling via G-protein–coupled receptors**

G-protein–coupled receptors (GPCRs) play a significant role in the regulation of cardiac function. Adrenergic (several subtypes of α- and β-adrenergic receptors) and muscarinic receptors are the most important myocardial GPCRs. These heptahelical receptors are coupled to three principal classes of heterotrimeric GTP-binding proteins, (Gs, Gq/G11, and Gi), which transduce the agonist-induced signal toward intracellular effectors such as enzymes and ion channels. All heterotrimeric G proteins consist of the subunits Gα and Gβγ, which upon receptor activation dissociate and independently activate intracellular signaling pathways.

Angiotensin II (Ang-II), endothelin-1 (ET-1), and alpha-adrenergic receptors are coupled to Gq/11 (which in turn activates phospholipase C), and promote cardiac hypertrophy upon agonist stimulation. Moreover, transgenic overexpression of these receptors as well as their downstream mediator Gq caused myocardial hypertrophy and subsequently leads to heart failure (Sakata et al., 1998; D'Angelo et al., 1997; Paradise et al., 2000; Koch et al., 2000).

β1-Receptors are the most abundant adrenergic receptor in heart which couple to Gs and activate adenylate cyclase (AC), ultimately causing positive chronotropic and inotropic effects in the heart. β2-Receptors are less abundant in the heart and couple to both Gs and Gi, providing an additional level of regulation of β-adrenergic signaling. Overexpression of β1-receptors in hearts of transgenic mice increased contractile function, but eventually caused impaired cardiac performance and cardiomyocyte hypertrophy (Engelhardt et al., 2002; Bisognano et al., 2000).

Cardiac muscarinic and β2-adrenergic receptors couple through Gi and inhibit AC, directly opposing Gs-dependent signaling. It was shown that myocardial Gi-proteins (Gi$_{\alpha 2}$) increased in human heart failure (Eschenhagen et al., 1992; Neumann et al., 1988) and basal AC activity is impaired, which is suggested that this mechanism may be involved in heart failure. Moreover, Gi is increased in hypertensive hypertrophy before the development of heart failure (Bohm et al., 1993). It was reported that the Gi antagonist pertussis toxin completely blocked hypertrophy in isolated cardiomyocytes stimulated by severe hypoxia (El Jamali et al., 2004). Furthur, it was demonstrated that under in vivo conditions, chronic β-adrenergic stimulation induces increased mRNA expression of Giα ( Eschenhagen et al., 1992) perhaps as a compensatory mechanism as chronic β-adrenergic stimulation induces cardiac hypertrophy (Frey and Olson, 2003).

## Molecular pathways for cardiomyocyte hypertrophy

### PI3K/Akt/GSK-3-dependent signaling

The precise intracellular signaling pathways that link the hypertrophic stimuli into the changes in cardiac gene expression, changes in nuclear transcription, and hypertrophic growth have not been fully established. Phosphoinositide 3-kinases (PI3Ks) are a family of enzymes which have both protein and lipid kinase activity. They are involved in many cellular functions, particularly cell growth, survival, and proliferation (Frey and Olson, 2003).

Several receptor tyrosine kinases, such as the IGF-1 receptor, as well as G protein-coupled receptors (GPCRs), including α- and β2-adrenergic receptors can

activate PI3Ks. It has been shown that PI3K is activated in pressure overload hypertrophy in a Gβγ -dependent fashion. In transgenic mice expressing constitutively active or dominant-negative mutants of PI3K in the heart, it was reported that the constitutively active PI3K transgene caused a larger heart associated with an increase in myocyte size compred with dominant negative mutants of PI3K (Shioi et al., 2000; Naga Prasad et al., 2000).

The serine/threonine kinase AKT (a.k.a. PKB) is one of the major targets of PI3K signaling. Activation of AKT increased cell size in Drosophila. Transgenic overexpression of Akt/PKB promotes significant cardiac hypertrophy in mice. It is reported that constitutive activation of Akt in skeletal muscle also causes hypertrophy (Shioi et al., 2002).

GSK-3 is defined as a direct target of AKT and AKT/PKB directly phosphorylates GSK-3β and blocks its activity. Various hypertrophic agonists including endothelin I, isoproterenol, and phenylephrine are associated with GSK-3β phosphorylation and inactivation of GSK-3 activity which is necessary for the hypertrophy process (Antos et al., 2002). GSK-3β appears to integrate signals of several hypertrophic pathways and its inactivation is critical and important for the development of many forms of cardiac hypertrophy (Frey et al., 2003). There is significant crosstalk between PI3K/AKT/GSK-3β and other hypertrophic pathways (Frey et al., 2003).

**MAPK pathways**

MAP kinase (MAPK) pathways provide an important link between external stimuli and the nucleus via phosphorylation and regulation of multiple transcription factors. Extracellularly responsive kinases (ERKs) are one of MAPKs subfamilies which are involved in cardiac hypertrophy. It was demonstrated that transgenic overexpression of MEK1, a MAPK kinase that activates ERK1/2, caused myocardial hypertrophy (Witt and Kimball, 2000).

**PKC and cardiac hypertrophy**

Both $\alpha_1$-adrenergic agonists and ET-1 (via Gq coupled receptors) stimulate the hydrolysis of the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2) in cardiac myocyte. This hydrolysis produces the 'second messenger" diacylglycerol which is the physiological activator of protein kinase C (PKC) (Sugden, 2001). Several studies have been shown that the various PKC isoforms are involved in cardiac hypertrophy and ventricular remodeling.

It was reported that activation of PKC by phorbol esters (such as PMA) mimics the prohypertrophic effects of phenylephrine-mediated PKC activation in cultured cardiomyocytes (Henrich and Simpson., 1988). The literature has concentrated on the cardiac actions of PKC$\alpha$, PKC$\beta$ and PKC$\varepsilon$, which activate ERK and inducing cardiomyocyte hypertrophy. Additionally PKC$\delta$ is linked to hypertrophy in some models (Steinberg, 2004). Transgenic overexpression of PKC-$\beta$ in transgenic hearts promotes cardiac hypertrophy and sudden death (Frey and Olson, 2003). It was reported that hypertrophic agonist, phorbol myristate acetate (PMA) induces nuclear

translocation of PKCα and that PKCα is both necessary and/or sufficient to induce certain features of cardiomyocyte hypertrophy including increased protein synthesis, increased protein/DNA ratio (P/D), increased cell surface and increased ANF expression in cultured neonatal rat cardiomyocytes (Braz et al., 2002).

### Effect of berberine on cardiac hypertrophy

Berberine has shown significant effect on inhibiting the development of cardiac hypertrophy in a pressure overload hypertrophic rat model system. Berberine treatment (oral administration of 10 mg/kg for 8 weeks) reduced the elevated heart weight, the myocardial cross-sectional area and the total protein of the left ventricle in the hypertrophic model rats. Moreover, the cardiac function was improved with berberine in this group (Hong et al., 2002). Thus, berberine may have therapeutic potential in the treatment of CHF (Hong et al., 2002).

Berberine has the potential to antagonise α-adrenergic receptors (Shen et al., 1997), and $\alpha_1$-adrenergic agonists are identified to be prime hypertrophic agents (Guo et al, 1998; Sugden and Clerk, 1998). Therefore, it is possible that berberine acts to prevent the hypertrophic effects of $\alpha_1$-adrenergic agonists (Hong et al., 2002). Berberine contributes to the prevention of cardiac hypertrophy development caused by pressure-overload but further studies are necessary to evaluate the effect of berberine on various factors (such as Ang-II, IGF-II and catecholamines release) which are related with cardiac hypertrophy (Hong et a.l, 2002).

In animal model studies [cardiac hypertrophy was induced by suprarenal abdominal aorta constriction in rats], Hong et al reported that berberine reduced

noradrenaline and adrenaline levels in plasma, and adrenaline levels left ventricular tissue. Therefore, it was suggested that berberine might down-regulate the sympathetic nervous system activity of rats after suprarenal abdominal aorta constriction (Hong et al., 2003).

Although several and different studies indicate important cardiovascular effects of hawthorn and berberine, the related mechanisms involved in the chronotropic activity of hawthorn and berberine have not been clearly defined. Our laboratory had shown that hawthorn extract preparations decreased the contraction frequency of neonatal murine cardiomyocytes independent of beta-adrenergic receptor blockade (Long et al., 2006). Therefore, we sought to further characterize the effect and mechanism of hawthorn and berberine on the contraction rate of cultured cardiomyocytes. We eventually developed and sought to test the hypothesis that components of hawthorn extract, as well as the purified compound berberine, exerted effects as muscarinic agonist-like agonists.

Chapter 2: Hawthorn (*Crataegus monogyna* Jacq.) extract exhibits

atropine-sensitive activity in a cultured cardiomyocyte assay

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## Abstract

Hawthorn (*Crataegus spp.*) plant extract is used as an herbal alternative medicine for the prevention and treatment of various cardiovascular diseases. Recently, it was shown that hawthorn extract preparations cause negative chronotropic effects in a cultured neonatal murine cardiomyocyte assay independent of beta-adrenergic receptor blockade (Long et al., 2006). The aim of this study was to further characterize the effect of hawthorn extract to decrease the contraction rate of cultured cardiomyocytes. To test the hypothesis that hawthorn is acting via muscarinic receptors, the effect of hawthorn extract on atrial versus ventricular cardiomyocytes in culture was evaluated.  As would be expected for activation of muscarinic receptors, hawthorn extract had a greater effect in atrial cells. Atrial and/or ventricular cardiomyocytes were then treated with hawthorn extract in the presence of atropine or himbacine.  Changes in the contraction rate of cultured cardiomyocytes revealed that both muscarinic antagonists significantly attenuated the negative chronotropic activity of hawthorn extract.  Using quinuclidinyl benzilate, L-[benzylic-4,4'-$^3$H] ([$^3$H]-QNB) as a radioligand antagonist, the effect of a partially purified hawthorn extract fraction to inhibit muscarinic receptor binding was quantified.  Hawthorn extract fraction 3 dose-dependently inhibited [$^3$H]-QNB binding to mouse heart membranes. Taken together, these findings suggest that decreased contraction frequency by hawthorn extracts in neonatal murine cardiomyocytes may be mediated via muscarinic receptor activation.

## Introduction

Hawthorn (*Crataegus oxyacantha*, *C. monogyna*, or related species) extracts have been recognized internationally as sources of medicine for centuries (Chang et al., 2005; Rigelsky et al., 2002; Fong et al., 2002). Hawthorn extract is used as an alternative therapy for cardiovascular disease in Europe and China (Chang et al., 2005; Rigelsky et al., 2002).  In Germany, Hawthorn has been approved for use as a therapeutic agent in the treatment of mild to moderate heart failure and coronary heart disease. Hawthorn extracts have been shown to decrease blood pressure, decrease total cholesterol, low-density lipoprotein cholesterol (LDL cholesterol), and triglyceride levels, and to possess cardiotonic, diuretic, vasorelaxant, and anti-atherosclerotic properties (Chang et al., 2005; Rigelsky et al., 2002; Fong et al., 2002; Veveris et al., 2004; Muller et al., 1999; Eaton et al., 2003; Kirakosyan et al., 2003; Habs,  2004; Chen et al., 1998; Celebi Kocyildiz et al., 2006; Zapatero, 1999).

Hawthorn extract preparations exhibit variable negative and positive chronotropic and positive inotropic cardiac properties, and have a chronodilatory property with respect to coronary blood flow. The extract also demonstrates anti-arrhythmic properties (Celebi Kocyildiz et al., 2006; Zapatero et al., 1999; Schussler et al., 1995a; Schussler et al., 1995b; Popping et al., 1995; Long et al., 2006). Hawthorn extract has been shown to have various effects on heart rate. In isolated guinea pig heart, contraction rate was influenced by the flavonoids with maximum positive chronotropic effects of up to 45%. Higher concentrations of luteolin-7-glucoside and hyperoside showed slight arrhythmias during the first 5 min of application (Schussler et al., 1995a). Monoacetyl vitexinrhamnoside concentration-dependently increased

heart rate. However, in anaesthetized animals of several species, hawthorn produced a decrease in heart rate (Schussler et al., 1995b). In rat cardiomyocytes, hawthorn extract was reported to have a potential anti-arrhythmic effect (Popping et al., 1995). Previous work by our group has shown that hawthorn extracts possess negative chronotropic effects in a cultured neonatal murine cardiomyocyte assay (Long et al., 2006).

The aim of this study was to investigate a possible mechanism underlying hawthorn's negative chronotropic activity. Hawthorn's effects differ from adrenergic properties associated with biogenic amines (Zapatero, 1999), and hawthorn's negative chronotropic effect is not due to beta-adrenergic receptor blockade (Long et al., 2006). Therefore, other receptors, ion channels or signaling molecules must be affected by hawthorn extract. One plausible explanation for this finding is that hawthorn extract decreases the contraction rate of cultured spontaneously-contracting cardiomyocytes via activation of muscarinic receptors. M2 muscarinic receptors are the predominant cholinergic-receptor subtype in the heart of mouse and other mammalian species associated with decreased cardiac contraction frequency (Ponicke et al., 2003; Dhein et al., 2001; Wang et al., 2004; Ford et al., 2003). M2-receptors are heterogeneously distributed in heart, with more receptors in atria than in ventricles (Brodde et al., 2001; Zang et al., 2004), and the highest concentrations of mRNA in the sinoatrial (SA) node (Hardouin et al., 1998).

Therefore, isolated atrial and ventricular cardiomyocyte cultures were chosen to assess the differential effects of hawthorn extract on these distinct cell populations. Non-selective (atropine) and selective (himbacine) M2 receptor antagonists were then

used to attempt to block hawthorn extract's effects to reduce contraction frequency. Finally, based on our observations, we investigated the ability of a sub-fraction of the hawthorn extract with significant negative chronotropic activity to block high affinity muscarinic receptor antagonist radioligand binding to murine heart membranes.

## Materials and Methods

### Materials

Quinuclidinyl benzilate, L-[benzylic-4,4'-$^3$H]; 37.0 Ci/mmol ([$^3$H]-QNB) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.). Trypsin and Minimum Essential Media (MEM) were purchased from Mediatec, Inc. (Herndon, VA, U.S.A.). Carbachol (Carbamylcholine chloride) and atropine (sulfate salt) were purchased from Sigma, and himbacine was from Acros Organics (NJ, U.S.A.). Tris-HCl was from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, U.S.A.) and $MgCl_2$ was purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). LH-20 chromatography resin was purchased from GE Healthcare Bio-Sciences AB. Timed pregnant Swiss-Webster mice (Charles River, NY, U.S.A.) and pups were housed and used in accordance with approved guidelines on the care and use of laboratory animals issued by Oregon State University's institutional animal care and use committee.

### Hawthorn extracts

Hawthorn extracts were prepared as previously described (Long et al., 2006) from two different sources: 1) tablet-derived extract was prepared from Heartcare® tablets standardized to 18.75% oligomeric procyanidins (Nature's Way Products, Inc, Springville, UT); 2) ethanolic extract was prepared from commercial dried Bulgarian

*Crataegus monogyna* Jacq. wild cultivar leaves and stems (purchased as *C. oxyacantha* from Voigt Global Distribution, Kansas City, MO, U.S.A.). A tablet-derived extract (HT) was prepared as follows; Heartcare® tablets were powdered using a clean mortar and pestle; the prepared powder was extracted with agitation in 100% ethanol for 1-2 h at 20-25˚C. After centrifugation the supernatant was recovered, filtered, and evaporated to yield a dark green oil that was dissolved in phosphate-buffered saline, pH 7.4 (PBS) and stored at –20˚C ( Long et al., 2006). Pure ethanol extraction limited the amount of tablet excipient present in the finished crude extract.

Crataegus leaf and stem extracts (HL) were prepared by non-optimized adaptation of methods described in the literature (Vierling et al., 2003). Dried leaves and stems were extracted with 50% aqueous ethanol for 48 h at 4˚ C. The ethanolic solution was filtered and evaporated under reduced pressure to yield an oily residue which was resuspended in PBS and stored at –20˚ C (Long et al., 2006).

Tablet-derived extracts possess residual excipients which make them less suitable for further separation.  Size exclusion chromatography (LH-20 resin) of the hawthorn leaf and stem (HL) extract--including assay for negative chronotropic activity in neonatal murine cardiomyocyte assays--was described previously (Long et al., 2006). Fraction 3 (F3) used in the present study was from the size exclusion chromatographic fractionation of the HL extract as described previously (Long et al., 2006).

## Ventricular and atrial neonatal murine cardiomyocyte cell cultures

Cultures of neonatal murine cardiomyocytes were prepared as previously described (Toraason et al., 1989; Wang et al., 1999). Briefly, hearts were removed in Hanks' balanced salt solution (HBSS) from 2-3 day old neonatal mice. Atrial and ventricular tissues were isolated by aid of a dissecting microscope, separately minced, and subjected to trypsin (0.1%) digestion at 37° C. Dissociated cells from each tissue were isolated at 10 min intervals throughout the digestion for up to 50 min. The dissociated cells were pooled, collected by centrifugation and resuspended in MEM prior to plating in 24-well plates (2cm²/well). Each well contained 500 µl of MEM and plates were incubated at 37° C in humidified 5% $CO_2$.

## Cardiomyocyte contraction rate measurement

After 2 days in culture, spontaneously beating cardiomyocytes were observed and recorded as previously described (Long et al., 2006). Contraction rate (beats per minute) of cardiomyocytes were recorded prior to the addition of phosphate-buffered saline, PBS (pH 7.4) vehicle, hawthorn crude extracts (HL and HT), HL extract fraction 3 (F3), and/or drugs. Changes in contraction rate were obtained by counting contractions per unit time exactly 2 minutes after addition of hawthorn extract and/or drugs. Spontaneously beating cardiomyocytes were pretreated for 2 min with 0.1 mM atropine or 0.01 mM himbacine prior to addition of hawthorn extract (HT or HL) or HL extract fraction 3 (F3), and the spontaneous contraction frequency was determined both before and 2 min after the addition of hawthorn extract and/or drugs. Contraction rate of cells analyzed for all experiments ranged from 20-160 beats/min in control vehicle.

## Cardiac membrane preparations

Membranes were prepared from female adult mouse heart as previously reported (Rashid et al., 2003) with minor modifications. Briefly, hearts were removed following animal sacrifice and homogenized on ice in hypotonic lysis buffer (10 mM Tris-HCl, pH 7.4, containing 250 mM sucrose) by Dounce homogenizer. The cell homogenate was centrifuged at low speed (500 x g for 10 min) to pellet nuclei and debris.  Low speed supernatants were pooled and centrifuged at 25,000 x g for 45 min at 4°C to pellet crude membranes. The resultant crude membrane pellet was resuspended in ice-cold 120 mM Tris-HCl, pH 7.4, and 40 mM $MgCl_2$, followed by storage at –80° C. Membrane protein concentrations were determined by the Lowry method (Peterson, 1979; Lowry et al., 1951) using bovine serum albumin as standard.

## Radioligand binding assay

Radioligand binding assay to quantify muscarinic receptor occupation was performed as described previously ((Rashid et al., 2003; Rashid et al., 2001) with minor modifications. All binding assays were initiated by adding 50 μl (0.25 mg/ml or 50 μg/assay) of crude membrane to samples on ice containing a final volume of 200 μl of assay buffer (60 mM Tris-HCl, 20 mM $MgCl_2$, and 0.1% ascorbic acid), varying concentrations of [³H]QNB (170-20,000 cpm/tube) as indicated, and PBS vehicle, 0.1 mM atropine, or hawthorn extract fraction 3 (F3).  Saturation isotherm binding experiments were performed in the presence of [³H]QNB at concentrations ranging from 0.05 to 5.0 nM, whereas competition binding experiments were performed in the presence of 0.4 nM of [³H]QNB and increasing concentrations of hawthorn extract fraction 3. Nonspecific binding was determined in the presence of

nonlabeled atropine sulfate, 0.1 mM.  After 30 min of incubation at 30˚C, binding reactions were terminated by rapid filtration through Whatman GF/C glass fiber filters using a Brandel cell harvester (Gaithersburg, MD, U.S.A.). Filters were washed 4 times with 3 ml of ice-cold wash buffer (50 mM Tris-HCl, pH 7.4). Filters were pre-wetted with ice-cold wash buffer to decrease non-specific binding of free radioligand. Radioactivity retained by filters was quantitated by liquid scintillation spectrometry.

## Analysis of data

All binding experiments were independently repeated three times in triplicate, and data are presented as the mean $\pm$ S.E.M.  Binding parameters were determined using Prism 3 software (GraphPad Software Inc. San Diego, CA, U.S.A.). Affinity ($K_D$) and maximal binding capacity ($B_{max}$) values were obtained from saturation isotherm specific binding data by nonlinear regression curve analysis using the standard equation for a rectangular hyperbola fitted to one-site. The concentration of hawthorn extract semi-purified fraction 3 required to inhibit 50% of specific [³H]QNB binding ($IC_{50}$) was calculated from linear regression analysis of a sigmoidal competition curve fitted to one-site. Results are displayed as percent specific binding representing the means $\pm$ S.E.M. of more than three separate experiments in triplicate.  Differences between means were tested for significance by student's t-test with $p < 0.05$.

## Results

## Effect of hawthorn extracts on spontaneous contraction rate of atrial compared to ventricular cardiomyocytes

Using spontaneously-beating isolated atrial and ventricular cardiomyocyte cultures, we determined the effects of hawthorn crude ethanolic extracts and a partially purified hawthorn extract fraction to directly affect contraction rate. In our previous study, we showed that size exclusion chromatography fractions of a crude hawthorn leaf and stem extract (HL) showed varying activities to increase or decrease contraction rate of cultured cardiomyocytes (Long et al., 2006). Among the HL extract size exclusion chromatography fractions, we previously showed that HL extract size exclusion fraction 3 (F3) displayed the greatest negative chronotropic activity towards a mix of atrial and ventricular cardiomyocytes (Long et al., 2006). In the current study, we determined the dose response curve for F3 to decrease the contraction rate specifically of atrial cardiomyocytes (Figure 2.1). F3 dose-dependently inhibited atrial cardiomyocyte contraction rate with an $IC_{50}$ value of 1.2 μg/μl. Additions of F3 extract at higher concentrations than those shown were limited by extract solubility and limited exact determination of $IC_{50}$ value.
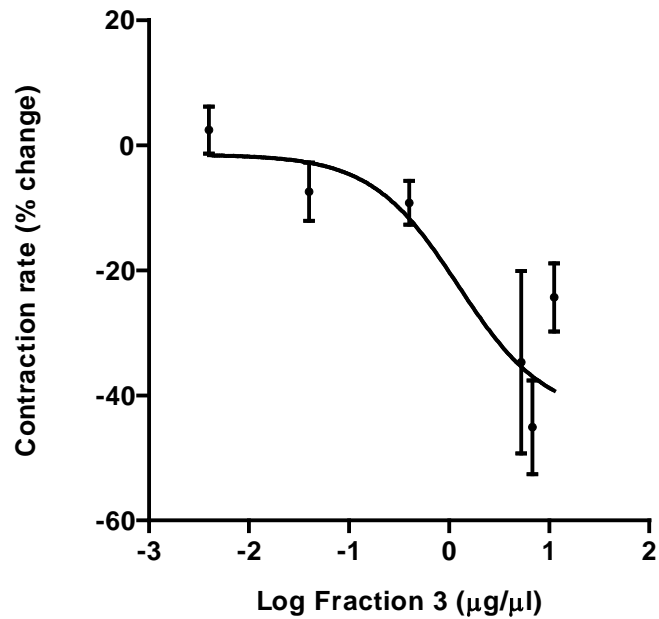
Figure 2.1 *Dose-response curve for the direct effect of fraction 3 on spontaneous contraction rate of cultured atrial cardiomyocytes.* Negative chronotropic effect of fraction 3 on atrial cardiomyocytes as a percentage of change in contraction rate was calculated as the difference between observed contraction rates in beats/min before and after treatment and considered as a percentage of the original contraction frequency for the observed cell. Means ± S.E.M. are shown.

PBS vehicle, carbachol (CCh), hawthorn leaf and stem crude extract (HL), tablet-derived hawthorn extract (HT), and F3 were assayed for effect on contraction rate. PBS vehicle had no effect on contraction rate as compared to the significant negative chronotropic activity of 100 nM carbachol, a known muscarinic receptor agonist and positive control. HL, HT and F3 all caused significant decreases in spontaneous contraction rate of atrial cardiomyocytes compared to the vehicle control (PBS) as shown in Figure 2.2. Based on previously published dose-response curves for HL and HT extracts (Long et al., 2006), we used a concentration of 0.2 mg/ml for the crude extracts which reproducibly decreased contraction rate without solubility problems or significant difficulties with arrythmias or cessation of beating. Carbachol concentration (100 nM) was chosen was based on its dose response curve to cause bradycardia in isolated mouse atria and to avoid the cessation of beating associated with higher concentrations (Stengel and Cohen, 2001).

The similarity of hawthorn's effect to that of the muscarinic agonist carbachol was investigated by determining the effect of atropine, a non-selective muscarinic antagonist, on the chronotropic response of atrial cardiomyocytes to hawthorn extracts. Atropine (At) at 0.1 mM had no significant effect on cardiomyocyte contraction rate compared to vehicle, as expected. However, pretreatment of atrial cardiomyocytes for 2 min with 0.1 mM atropine completely attenuated the negative chronotropic effect of HL, HT, and F3 hawthorn extracts (Figure 2.2). The $K_D$ of atropine for M2 receptors is 0.4 nM (Rashid et al., 2003).
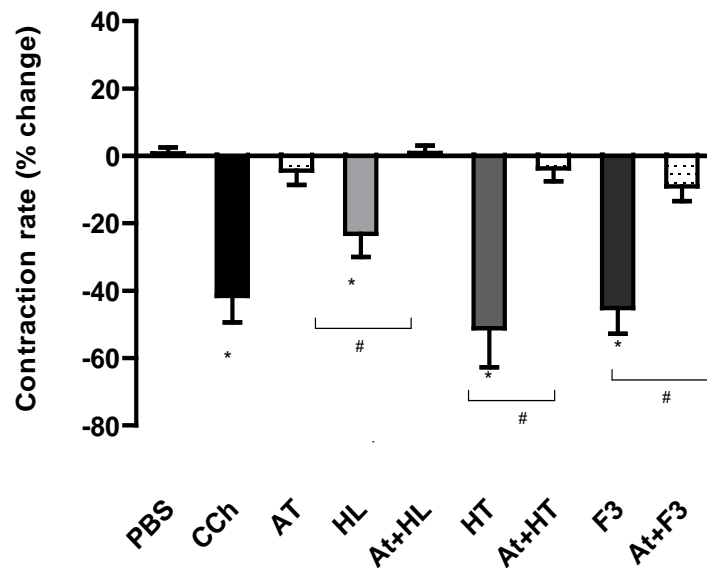
Figure 2.2 *Effect of atropine pretreatment on direct negative chronotropic response of hawthorn extracts in neonatal murine atrial cardiomyocytes*. Cultured neonatal atrial cardiomyocytes were treated with PBS, 100 nM carbachol (CCh), 0.2 mg/ml hawthorn leaf and stems extract (HL), 0.2 mg/ml tablet-derived hawthorn extract (HT), and 6.8 µg/ml fraction 3 (F3). Atrial cardiomyocytes pretreated for 2 min with 0.1 mM atropine (At). After pretreatment, the contraction rate was determined before and after the addition of hawthorn preparations at the concentrations indicated. Chronotropic response is shown as percent change in contraction rate calculated as the difference between observed contraction rates in beats/min prior to and after treatment, and considered as a percentage of the original contraction frequency for the observed cell. Means + S.E.M are shown. * indicates significant difference at $p<0.05$ in average values as compared to control (PBS). # indicates significant difference at $p<0.05$ between treatment groups in the absence and presence of atropine.

To further verify the specificity of atropine blockade on hawthorn-induced negative chronotropic activity, we also pretreated the atrial cardiomyocyte preparations for 2 min with 0.01 mM himbacine, a selective M2-receptor antagonist. The $K_D$ of himbacine for M2 receptors is 6.9 nM (Miller et al., 1992). HL, HT and F3 hawthorn extracts produced no significant negative chronotropic activity compared to PBS vehicle in himbacine pre-treated cells (Figure 2.3).
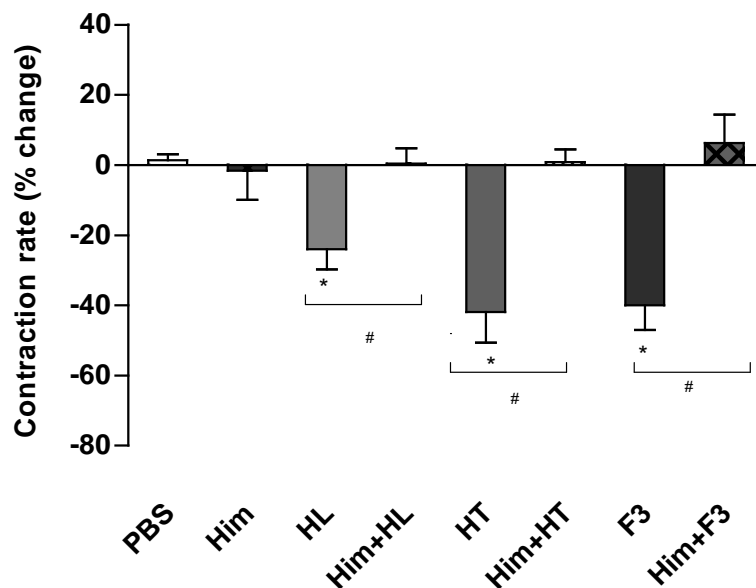


Figure 2.3 *Himbacine-sensitive activity of hawthorn extracts on atrial cardiomyocytes.* Cultured neonatal atrial cardiomyocytes were treated with PBS, 0.2 mg/ml hawthorn leaf and stems extract (HL), 0.2 mg/ml tablet-derived hawthorn extract (HT), and 6.8 µg/ml fraction 3 (F3). Atrial cardiomyocytes were pretreated for 2 min with 0.01 mM himbacine (Him), and the contraction frequency determined before and after addition of different hawthorn preparations at the concentrations indicated. Chronotropic response is shown as percent change in contraction rate calculated as the difference between observed contraction rates in beats/min prior to and after treatment and considered as a percentage of the original contraction

frequency for the observed cell. Means + S.E.M are shown. * indicates significant difference at $p<0.05$ in average values as compared to control (PBS). # indicates significant difference at $p<0.05$ between treatment groups in the absence and presence of himbacine.

## Effect of hawthorn extracts in ventricular cardiomyocytes

In isolated ventricular cardiomyocytes, carbachol (CCh), HT and F3 extracts produced significant negative chronotropic effects (P<0.05) compared to PBS. HL extract at 0.2 mg/ml caused a significant (and dose dependent) increase in contraction rate of ventricular cardiomyocytes as shown in Figure 2.4 (and data not shown).

Pretreatment of ventricular cardiomyocytes for 2 min with 0.1 mM atropine or 0.01 mM himbacine as above resulted in a complete blockade of the negative chronotropic effects of HT and F3 extracts (Figure 2.4).

## Binding affinity

A direct interaction of hawthorn extract components with muscarinic receptors was investigated using a competitive radioligand binding assay in adult mouse heart membranes. $K_d$ and $B_{max}$ values for [$^3$H]-QNB binding are the same in membranes from adult and neonatal murine heart (Evans et al., 1985). Therefore, we used adult mouse heart membranes due to the increased abundance of tissue needed for the competitive binding experiments.
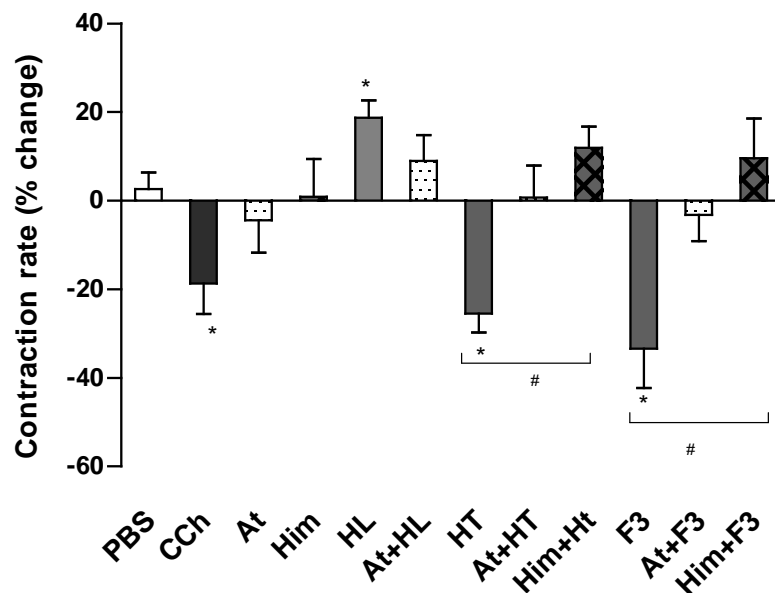
Figure 2.4 *Effect of atropine or himbacine pretreatment on chronotropic response of hawthorn extracts in neonatal murine ventricular cardiomyocytes.* Cultured neonatal ventricular cardiomyocytes were treated with PBS, 100 nM carbachol (CCh), 0.2 mg/ml hawthorn leaf and stems (HL), 0.2 mg/ml tablet-derived hawthorn extract (HT), and 6.8 µg/ml fraction 3 (F3). Ventricular cells pretreated with 0.1 mM atropine (At) or 0.01 mM himbacine (Him) for 2 min, then different hawthorn preparations were added to the cardiomyocytes at the concentrations indicated. Chronotropic response is shown as percent change in contraction rate calculated as the difference between observed contraction rates in beats/min prior to and after treatment and considered as a percentage of the original contraction frequency for the observed cell. Means + S.E.M are shown. * indicates significant difference at $p < 0.05$ in average values as compared to control (PBS). # indicates significant difference at $p < 0.05$ between treatment groups in the absence and presence of atropine or himbacine.

In our hands, radiolabeled muscarinic antagonist [³H]QNB bound to a single saturable site in mouse heart membranes with a $B_{max}$ value of 71.4 ± 4.4 fmol/mg and a $K_D$ value of 0.32 ± 0.07 nM. F3 extract was evaluated for ability to displace 0.4 nM [³H]-QNB binding in mouse heart membranes. Competition binding experiments demonstrated that F3 extract displaced [³H]-QNB binding in a dose dependent manner with an $IC_{50}$ value greater than or equal to 0.4 µg/µl as shown in Figure 2.5. Exact determination of $IC_{50}$ value was limited by extract solubility.
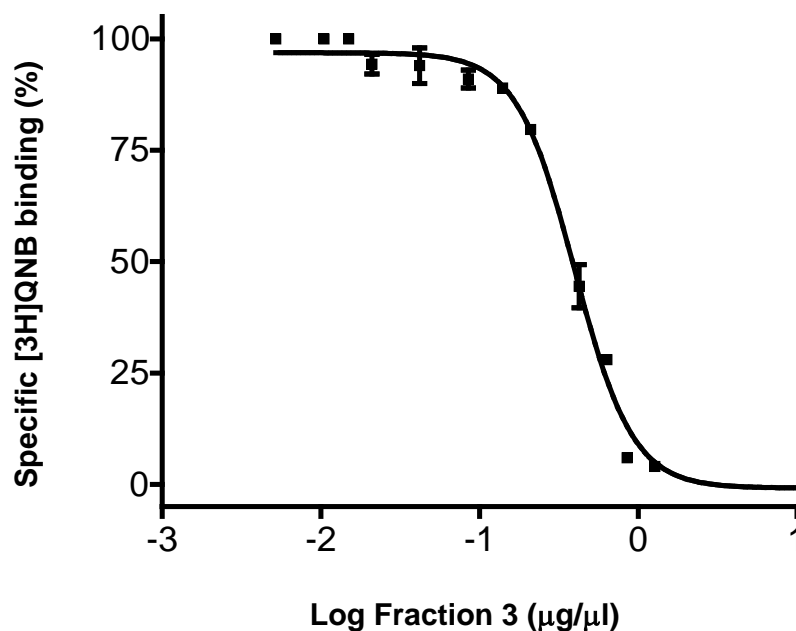


Figure 2.5 *Competition binding profile of fraction 3 against specific [³H]QNB binding in mouse heart membranes*. Indicated concentrations of fraction 3 were co-incubated with 0.4 nM [³H]QNB in radioligand competition binding assays with adult mouse heart membranes. Radioactivity retained by filters is quantitated and nonspecific binding in the presence of 0.1mM atropine is subtracted from all data points. Data represent the mean ± S.E.M. of more than three determinations, each performed in triplicate and expressed as the percentage of specific binding.

## Discussion

The main findings of this study are: 1) HL extract has significant negative chronotropic activity towards atrial cardiomyocytes, but significantly increases the contraction rate of ventricular cells; 2) the direct negative chronotropic effects of HT and F3 extracts, similar to the positive control carbachol (CCh), were greater in atrial cardiomyocytes than in the ventricular cells; 3) atropine and himbacine, muscarinic receptor antagonists, blocked the negative chronotropic effects of hawthorn extracts; and 4) competition binding studies suggest that F3 extract contains a potential ligand(s) for muscarinic receptors in mouse heart membranes. In summary, these data support our hypothesis that hawthorn extracts caused a decrease in contraction rate of cardiomyocytes via muscarinic receptor activation. To our knowledge, this is the first report to show that a muscarinic agonist-like activity may be present in hawthorn extracts.

The reported effects of hawthorn extract in human subjects are not inconsistent with muscarinic-like effects. The principal effect of muscarinic receptors stimulation in the coronary vasculature is vasodilation (Dhein et al., 2001) and acetylcholine produces dilation of essentially all vascular beds, including coronary vasculature. Hawthorn extract induced concentration-dependent relaxation in rat isolated mesenteric arteries that was similar to acetylcholine, the endothelium-dependent vasodilator (Chen et al., 1998). It has also been shown that application of hawthorn extract increases coronary blood flow in the isolated heart as well as in whole animal models (Veveris et al., 2004).

In rat cardiomyocytes, it was reported that hawthorn extract has a potential anti-arrhythmic activity (Rigelsky and Sweet, 2002). Stimulation of muscarinic receptors can significantly reduce malignant arrhythmias during acute myocardial ischemia (De Ferrari et al., 1992). The general finding is that low concentrations of muscarinic agonists decrease heart rate, while high concentrations might under certain circumstances increase heart rate (Dhein et al., 2001).

The adverse effects associated with hawthorn extract within the therapeutic dose ranges are mild and tolerable and include palpitation, sweating, rash, headache, dizziness, vertigo, agitation, sleepiness, and gastrointestinal symptoms. Larger doses of hawthorn extract produce arrhythmia, hypotension and sedation (Chang et al., 2005; Rigelsky and Sweet, 2002). Some of these effects also were observed with the activation of muscarinic receptors; for example, carbachol-induced sedation is mediated by activation of M2 muscarinic receptors and is completely blocked by pretreatment with M2-muscarinic antagonist, methoctramine (Ma et al., 2001). Additionally, some of hawthorn's effects or adverse effects are likely not associated with activation of muscarinic receptors, but may be due to the combined activity of other cardioactive components within crude hawthorn preparations.

The similarity of hawthorn's effects to those of muscarinic agonists raises the possibility that hawthorn extract possesses functional M2-selective muscarinic agonists.  In our experiments, a non-subtype selective M-receptor antagonist, atropine, and a more M2 selective antagonist, himbacine, both block hawthorn's effect on contraction frequency of atrial and ventricular cultured neonatal murine cardiomyocytes.  The greater sensitivity of atrial cells compared with ventricular cells

to carbachol and hawthorn extracts might be explained by the much greater density of M2 muscarinic receptors (Zang et al., 2004).

However, given the lack of pharmacological selectivity of most muscarinic agonists for the M1 and M2 muscarinic receptor subtypes, the most likely explanation is that hawthorn extract possesses a non-selective muscarinic-type agonist, perhaps one similar to classic muscarinic receptor natural product agonist, pilocarpine. Sabcomeline and xanomeline are functional M1 receptor agonists, but both compounds possess high affinity at M2 receptors. In isolated mouse atria it has been shown that xanomeline and sabcomeline can both induce a negative chronotropic effects (Stengel and Cohen, 2001).

Unlike hawthorn tablet (HT) or fraction 3 (F3), hawthorn leaf extract (HL) caused a direct significant and dose-dependent increase in contraction frequency of ventricular cardiomyocytes. Hawthorn extract likely contains several compounds with varying activities on isolated cardiomyocytes. Initial separation of hawthorn extract by size exclusion chromatography yielded 10 fractions with varying activities in isolated cardiomyocytes. Fraction 1 displayed clear positive chronotropic activity towards isolated cardiomyocytes (Long et al., 2006). It is possible that fraction 1 from hawthorn leaf extract contains a sympathomimetic compound(s) that induces dose-dependent positive chronotropic activity in the ventricular cardiomyocytes. In the heart, positive chronotropic effects of catecholamines are mediated by beta-adrenergic receptors (Santos and Spadari-Bratfisch, 2006). In isolated guinea pig heart, heart rate was influenced by flavonoids with maximum positive chronotropic effects of up to 45%. Higher concentrations of luteolin-7-glucoside and hyperoside showed slight

arrhythmias during the first 5 min of application (Schussler et al., 1995a). It was reported that possible beta-adrenergic activities of flavonoids could be excluded by the addition of propranolol (Schussler et al., 1995b). The number of β-adrenoceptors in the heart is quite evenly distributed in atrial and ventricular tissues, but the β1:β2-adrenoceptor ratio slightly differs between atrial and ventricular tissues. In contrast to β-adrenoceptors, regional differences exist for the distribution of M2-receptors in the heart; the number of M2- receptors is significantly higher in the atria than it is in the ventricular tissues (Brodde et al., 2001). Thus, the difference in atrial and ventricular isolated cardiomyocyte effects of hawthorn leaf extract may be a result of lower numbers of M2 receptors in the ventricular cells which unmasks a coincident positive chronotropic effect of coexisting flavonoids in the extract.

HL size exclusion fraction 3 (F3) dose-dependently inhibited [$^3$H]-QNB binding with an $IC_{50}$ value of at least 0.4 μg/μl; F3 inhibited contraction with an $IC_{50}$ value of at least 1 μg/μl. Calculation of $IC_{50}$ values in both assays was limited by the solubility of higher concentrations of F3 extract fraction. However, both $IC_{50}$ values are the same within the range of error, suggesting that interaction with muscarinic receptors is responsible for the reduction in cardiomyocyte contraction rate in our system.

Chemically, hawthorn preparations have many different chemical constituents, including flavonoids (rutin, hyperoside, vitexin rhamnoside), proyanidins, other polyphenols, crataegolic acid, ursolic and oleanolic acids (Chang et al., 2005 ;Fong and Bouman, 2002 ;Veveris et al., 2004 ;Celebi Kocyildiz et al., 2006 ;Tankanow et al., 2003; Quetttier-Deleu et al., 2003; Ju, 2005). Additional minor constituents are

still being identified in hawthorn preparations. After fraction 3 was utilized for a

variety of activity assays, insufficient material remained for a thorough investigation

of the responsible cardioactive component.   Therefore, a larger scale extraction and

further purification steps will be necessary to isolate, purify and identify the

compound(s) which contribute to the negative chronotropic effect of hawthorn

extracts in neonatal murine cardiomyocytes and which bind to muscarinic receptors.

Chapter 3:  Berberine possesses muscarinic agonist-like properties in

cultured rodent cardiomyocytes


Satin Salehi and Theresa M. Filtz

Abstract

Berberine exhibits variable positive and negative chronotropic effects in different species. The first aim of the present study was to investigate the effect of berberine in a cultured neonatal murine cardiomyocyte assay. Our study demonstrates that berberine has significant negative chronotropic activity on cultured neonatal cardiomyocytes that was not an effect of beta-adrenergic receptor blockade.

Pertussis toxin (PTX), a Gi/o protein inhibitor, blocked the negative chronotropic activity of berberine.

Muscarinic, adenosine, opioid and α-receptors are coupled by a G-protein (Gi/o) to adenylyl cyclase in an inhibitory fashion. Activation of these receptors are primarily responsible for negative chronotropic effects in heart, thus we hypothesized that berberine may be acting through these receptors to decrease contraction rate of cardiomyocytes. For this purpose, we studied the effects of the muscarinic-receptor antagonists, atropine, himbacine, or AF-DX 116 on the negative chronotropic activity of berberine. Muscarinic antagonists completely blocked the effect of berberine on contraction rate of cardiomyocytes, whereas the bradycardic effect of berberine was not opposed by antagonists to opioid, adenosine or α-adrenergic receptors.

Using [$^3$H]QNB as a radioligand, we demonstrated that berberine bound to muscarinic receptors of adult mouse heart membranes with relatively high affinity ($K_i = 2.4 \times 10^{-6}$ or 2.4 µM). Furthermore, berberine dose-dependently inhibited [$^3$H]QNB binding to muscarinic M2 receptors exogenously expressed in HEK 293 cells with an affinity of 4.9 µM. Therefore, the findings of the present study suggest that

berberine has muscarinic agonist effects in cultured neonatal murine cardiomyocytes, potentially explaining some reported cardio-physiological effects of berberine.

## Introduction

Berberine [5,6-dihydro-9,10-dimethoxy-benzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium], an alkaloid isolated from a variety of plants such as *Berberis aquifolium* and *Berberis aristata*, has a long history in traditional Chinese medicine. Berberine has been found to have various pharmacological actions, including antidiarrheal, antibiotic, antitumor, anti-inflammatory, vasorelaxant and antiproliferative effects (Lau et al., 2001; Cheng et al., 2006; Yang et al., 2006). In guinea-pig ileum, berberine enhanced contractility by increasing acetylcholine release from postganglionic parasympathetic nerve terminals, increasing acetylcholine retention through an inhibition of cholinesterase activity and blocking $\alpha_2$–adrenoceptors (Shin et al., 1993). It was suggested that the anti-amnesic effect of berberine may be related to an increase in peripheral and central cholinergic neuronal system activity (Peng et al., 1997). Animal and clinical studies have suggested a number of beneficial effects of berberine on cardiovascular system. It is reported that berberine and its derivatives have positive inotropic and antiarrhythmic properties. Recently, the efficacy and safety of berberine was assessed for the treatment of chronic heart failure, and it was shown that berberine increased left ventricular ejection fraction and exercise capacity, improved quality of life, and decreased mortality in patients with congestive heart failure (Lau et al., 2001; Yang et al., 2006; Zeng et al., 2003).

Berberine exhibits variable positive and negative chronotropic effects in different species. Berberine stimulated the in situ dog heart and produced tachycardia, and in smaller doses stimulated the isolated heart of the rabbit and the frog (Sabir and Bhide, 1971). On the other hand, it was reported that berberine produced a concentration-dependent negative chronotropic effect in spontaneously beating right atria of guinea pig and rabbit sinoatrial cells (Shaffer, 1985; Riccioppo neto, 1993). Berberine did not affect the positive chronotropic activity of isoprenaline in rabbit right atria suggesting that β–adrenergic receptor blockade was not involved (Riccioppo neto, 1993). It is not clear what mechanism produced the reduction in spontaneous rate of atrial cells (Shaffer, 1985).

The aim of this study was to discover a possible mechanism underlying berberine's bradychardia. Here, the direct effect of berberine in cultured neonatal murine cardiomyocytes showed that berberine significantly decreased contraction frequency of neonatal murine cardiomyocytes which was not through beta-adrenergic receptor blockade. Therefore, other receptors, ion channels or signaling molecules must be affected by berberine.

One plausible explanation for this finding is that berberine's negative chronotropic effect is via activation of muscarinic receptors. M2 muscarinic receptors are the predominant cholinergic-receptor subtype in the heart of mouse and other mammalian species associated with decreased cardiac contraction rate (Ponicke et al., 2003; Dhein et al., 2001; Wang et al., 2004). Additionally, Schmeller and co-workers have convincingly demonstrated that in porcine brain, berberine clearly binds to muscarinic receptors with an $IC_{50}$ of 1 µM (Schmeler, 1997). The effect of d-

berbamine on [³H]QNB binding to the M-cholinergic receptors of rat brain showed a high affinity with Ki values of $1.9 \times 10^{-7}$ (Hou and Liu, 1988).

In cardiac atrial cells, muscarinic agonists increase a voltage-sensitive $K^+$ current through activation of M2- type muscarinic receptors coupled to the pertussis toxin (PTX)-sensitive G-protein Gi/o (Sorota et al., 1985; Reisine, 1990). In the present study, we characterized the pharmacological sensitivity of berberine to muscarinic receptor antagonists and PTX in attempts to define the mechanism of action of berberine in isolated cardiomyocytes.

## Materials and Methods

### Preparation of neonatal murine cardiomyocyte cultures

Cultures of neonatal murine cardiomyocytes were prepared as previously described (Toraason et al., 1989; Wang et al., 1999). Briefly, hearts were removed in Hanks' balanced salt solution (HBSS) from 2-3 day old neonatal mice, finely minced, and subjected to trypsin (0.1%) digestion at 37°C. Dissociated cells from minced tissue were isolated at 10 min intervals throughout the digestion for up to 50 min. The dissociated cells were pooled, collected by centrifugation and resuspended in Minimum Essential Media (MEM) prior to plating in 24-well plates (2 cm²/well) in 500 µl of MEM and incubation at 37°C in humidified 5% $CO_2$ air.

### Cardiomyocyte contraction rate measurement

After 2 days in culture, spontaneously beating cardiomyocytes were observed and recorded as previously described (Long et al., 2006). Contraction rate (beats per minute) of cardiomyocytes was recorded prior to the addition of phosphate-buffered saline, pH 7.4 (PBS) vehicle, or drugs. Changes in contraction rate were obtained by

counting contractions per unit time exactly 2 minutes after addition of drugs.

Spontaneously beating cardiomyocytes were pretreated for 2 min with atropine,

himbacine or other drugs prior to addition of berberine, and the spontaneous

contraction frequency was determined both before and 2 min after the addition of

berberine and/or drugs. Contraction rate of cells analyzed for all experiments ranged

from 20 to160 beats/min in control vehicle.

## Pertussis toxin pretreatment

After one day in culture, cardiomyocytes were incubated for 24 h with 100 ng/ml

PTX.  The spontaneous contraction rate of cardiomyocytes was recorded as above.

## Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified

Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and

100 µg/ml streptomycin in an atmosphere of 5% $CO_2$.  HEK 293 cells were

transfected by the calcium phosphate precipitation method using, QIAGEN®

(Valencia, CA) midiprep-purified DNA plasmid (10 µg total) as described previously

(Zhang et al., 2006).

## Cardiac membrane preparations

Membranes were prepared from female adult mouse heart as described previously

(Rashid et al., 2003) with minor modifications. In brief, hearts were removed

following animal sacrifice and homogenized on ice in hypotonic lysis buffer (10 mM

Tris-HCl, pH 7.4, containing 250 mM sucrose) by Dounce homogenizer. The cell

homogenate was centrifuged at low speed ($500 \times g$ for 10 min) to pellet nuclei and

debris.  Low speed supernatants were pooled and centrifuged at $200,000 \times g$ for 45

min at 4°C to pellet crude membranes. The resultant crude membrane pellet was resuspended in ice-cold 120 mM Tris-HCl, pH 7.4, and 40 mM $MgCl_2$, followed by storage at –80°C.

## Membrane preparations of transfected HEK 293 cells

Forty-eight hours after transfection, HEK 293 cells were washed with ice-cold PBS, scraped and harvested at $500 \times g$ for 5 min. Crude membranes were prepared as above and suspended in binding buffer (25 mM Tris, pH 7.4, 10 mM $MgCl_2$, 0.1 mM PMSF, 1 µM pepstatin A, 10 µM leupeptin), followed by storage at –80°C. Membrane protein concentrations were determined by the Lowry method (Peterson, 1979; Lowry et al., 1951) using bovine serum albumin as standard.

## Radioligand Binding Assay

Radioligand binding assay to quantify muscarinic receptor occupation was performed as described previously (Rashid et al., 2003; Rashid et al., 2001) with minor modifications. All binding assays were initiated by adding 50 µl (0.25 mg/ml or 50 µg/assay) of crude membrane to samples on ice containing a final volume of 200 µl of assay buffer (60 mM Tris-HCl, 20 mM MgCl2, and 0.1% ascorbic acid for cardiac membranes or 25 mM Tris, pH 7.4, 10 mM $MgCl_2$ for transfected HEK 293 membrane), varying concentrations of [³H]QNB, 0.1 mM atropine, or berberine. Saturation isotherm binding experiments were performed in the presence of [³H]QNB at concentrations ranging from 0.05 to 5.0 nM, whereas competition binding experiments were performed in the presence of 0.4 nM of [³H]QNB and increasing concentrations of berberine. Nonspecific binding was determined in the presence of nonlabeled atropine sulfate, 0.1 mM. After 30 min of incubation at 30°C, binding

reactions were terminated by rapid filtration through No. 32 glass fiber filters (Whatman, Sanford, ME) using a Brandel cell harvester (Gaithersburg, MD). Filters were washed 4 times with 3 ml of ice-cold wash buffer (50 mM Tris-HCl, pH 7.4 for cardiac membrane or 25 mM Tris, pH 7.4, 10 mM $MgCl_2$ for transfected HEK 293 membrane). Filters were presoaked in 0.1% polyethyleneimine (PEI) to decrease non-specific binding of free radioligand. Radioactivity retained by filters was quantitated by liquid scintillation spectrometry.

## Data Analysis

All binding experiments were independently repeated more than three times in triplicate, and data are presented as the mean ± S.E.M.  Binding parameters were determined using Prism 5 software (GraphPad Software Inc. San Diego, CA, U.S.A.). Affinity ($K_D$) and maximal binding capacity ($B_{max}$) values were obtained from saturation isotherm specific binding data by nonlinear regression curve analysis using the standard equation for a rectangular hyperbola fitted to one-site. The concentration of berberine required to inhibit 50% of specific [$^3$H]QNB binding ($IC_{50}$) was calculated from regression analysis of a sigmoidal competition curve fitted to one-site. Results are displayed as percent specific binding representing the means ± S.E.M. of more than three separate experiments in triplicate. The dissociation constants of the competitors (berberine, carbachol) were calculated from the relationship  $K_i = IC_{50} /(1+ D/K_D)$ where $K_D$ is the dissociation constant for [$^3$H]QNB and $IC_{50}$  is the given concentration of competitor producing 50 percent inhibition of [$^3$H]QNB and D is the radioligand concentration (Hui et al., 1991).

Statistical significance of the data was evaluated using students't-test for the comparison of two groups and ANOVA followed by Tukey's test for comparison of more than three groups. P value of less than 0.05 was considered statistically significant.

Materials

[³H]QNB (quinuclidinyl benzilate, L-[benzilic-4,4'-3H]-; 37.0Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.).Trypsin, Minimum Essential Media and Dulbecco's modified Eagle's medium were purchased from Mediatec, Inc. (Herndon, VA, U.S.A.). Atropine (sulfate salt) was purchased from Sigma, and himbacine was from Acros Organics (NJ, U.S.A.). AF-DX 116, pirenzepine dihydrochloride, and CGS 15943 were purchased from Tocris (Ellisville, MO, U.S.A.). Phentolamine mesylate and naloxone hydrochloride were from MP Biomedicals, LLC (Solon, OH, U.S.A.).

Tris-HCl was from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, U.S.A.) and $MgCl_2$ was purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Berberine sulfate was from Tokyo Kaser Kogyo Co. (Tokyo, Japan) and epinephrine, methionine enkephalin, clonidine hydrochloride, and 2-chloroadenosine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PTX was from List Biological Laboratories, Inc. (Campbell, CA, U.S.A.). A plasmid containing the porcine M2 receptor (pCDPS-m2-MuscAR) was kindly provided by Dr. Mark Leid (Oregon State University, Corvallis, OR). Timed pregnant Swiss-Webster mice (Charles River, NY, U.S.A.) and pups were housed and used in accordance with approved guidelines on

the care and use of laboratory animals issued by Oregon State University's institutional animal care and use committee.

## Results

## Effect of berberine on spontaneous contraction rate of cardiomyocytes

In this study, we determined the dose response curve for berberine to decrease the contraction rate of neonatal murine cardiomyocytes (Figure 3.1A). Berberine displayed an inhibitory effect on cardiomyocyte contraction rate with an $EC_{50}$ of 0.1 µM. A concentration of 20 µM for maximal effect was used in subsequent experiments. We compared the concentration response of carbachol and berberine in cardiomyocytes. Berberine produced bradycardia ($EC_{50}$= 0.1 µM) similar to carbachol ($EC_{50}$= 0.3 µM) in the neonatal murine cardiomyocytes (Figure 3.1A, 3.1B and table 3.1).
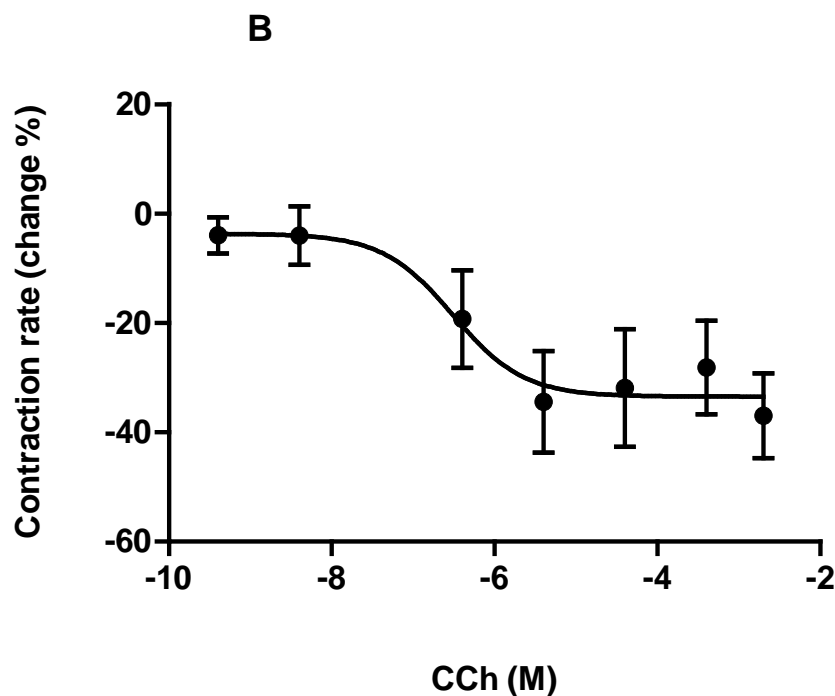
**A**

**B**
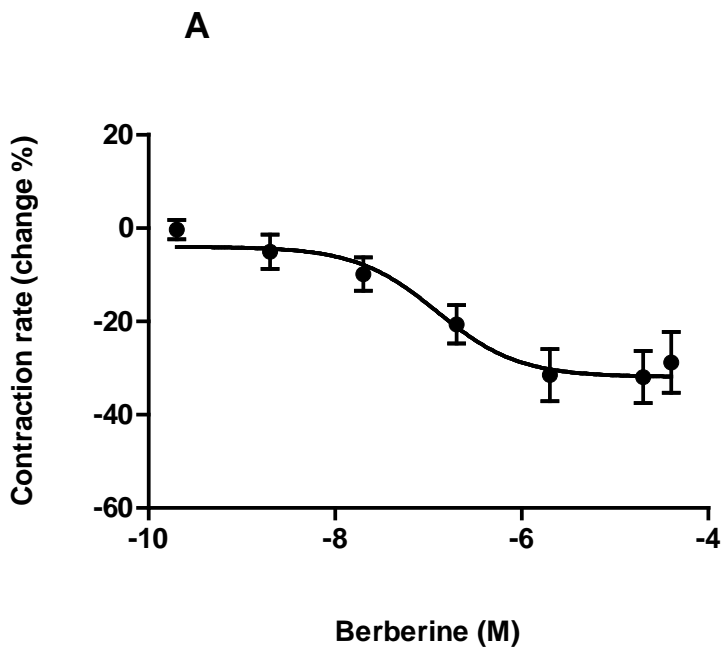


Figure 3.1 A) *Dose-response curve for the direct effect of berberine on the spontaneous contraction rate of cultured cardiomyocytes.* The negative chronotropic effect of berberine on cardiomyocytes was calculated as the difference between observed contraction rates in beats/min before and after treatment as a percentage of the original contraction frequency. Means ± S.E.M. are shown, $EC_{50} = 0.1$ µM. B) *Dose-response curve for the direct effect of carbachol (CCh) on the spontaneous contraction rate of cultured cardiomyocytes.* The negative chronotropic effect of carbachol on cardiomyocytes was calculated as in A, $EC_{50} = 0.3$ µM.

Table 3.1 Binding affinities of berberine and carbachol to M2 muscarinic receptors in the mouse heart and transfected HEK 293 membrane preparations using [³H]QNB as a radioligand

| Drug | $EC_{50}$ µM | $E_{MAX}$ (% decrease) | Adult mouse heart $K_i$ (M) | M2R transfected HEK cells $K_i$ (M) |
|---|---|---|---|---|
| Berberine | $0.1 \pm 0.1$ | $31.9 \pm 3.3$ | $2.4 \pm 0.8 \times 10^{-6}$ | $4.9 \pm 0.7 \times 10^{-6}$ |
| Carbachol | $0.3 \pm 0.1$ | $33.4 \pm 4.2$ | $1.78 \pm 0.3 \times 10^{-5}$ | $1.64 \pm 0.6 \times 10^{-5}$ |

To examine whether berberine possesses adrenoceptor blocking activity, we investigated the effect of berberine on the positive chronotropic response of cardiomyocytes to epinephrine. Pretreatment of cells for 20 min with 20 µM berberine had no significant effect on the positive chronotropic activity of 20 µM epinephrine (Figure 3.2).
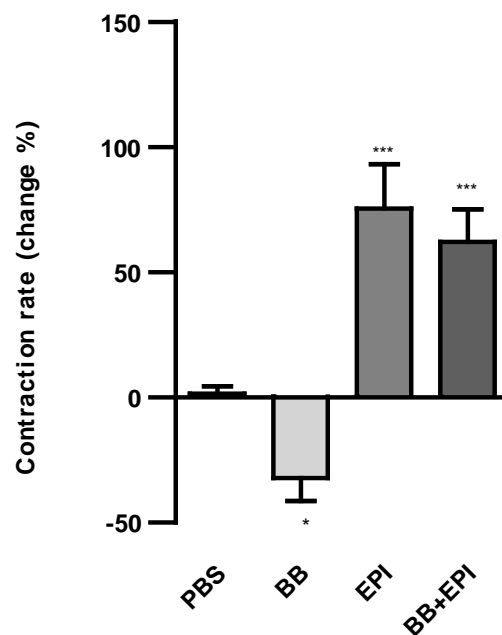


Figure 3.2 *Comparison of cardioactive drugs with berberine and PBS vehicle.* Cultured neonatal cardiomyocytes were treated with phosphate buffered saline (PBS), 2 µM carbachol (CCh), 20 µM epinephrine (EPI), or 20 µM berberine (BB). Cardiomyocytes pretreated for 2 min with 20 µM berberine and then 20 µM epinephrine (BB+EPI) was added to the cells. The contraction rate was determined before and after the addition of berberine at the concentrations indicated. Chronotropic response is calculated as the difference between observed contraction rates in beats/min prior to and after treatment as a percentage of the original contraction frequency. Means + S.E.M are shown. * and *** indicate significant difference at $p < 0.05$ and $p < 0.001$ respectively in average values as compared to control (PBS).

PTX experiments

In cardiac atrial cells, muscarinic agonists increase a voltage-sensitive $K^+$ current through activation of M2- type muscarinic receptors coupled to the pertussis toxin (PTX)-sensitive G-protein Gi/o (Reisine, 1990). This muscarinic agonist regulation of the potassium current is inhibited in isolated atrial myocytes by pretreatment with pertussis toxin (PTX) which selectively modifies $G\alpha_i$ family G proteins (Smrcka, 2008; Leaney and Tinker, 2000). The similarity of berberine's effect to that of a muscarinic agonist was investigated by pretreatment with PTX to abolish the chronotropic response of cardiomyocytes to berberine.

Preincubation of neonatal murine cardiomyocytes with a low PTX concentration (100 ng/ml) suppresses the negative chronotropic effects of berberine (Figure 3.3).
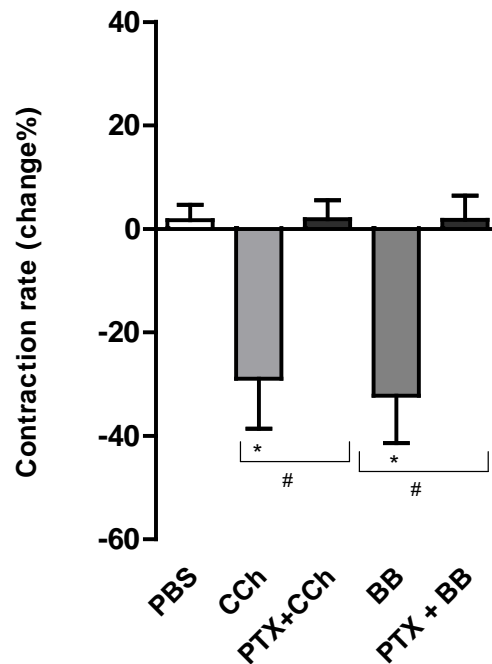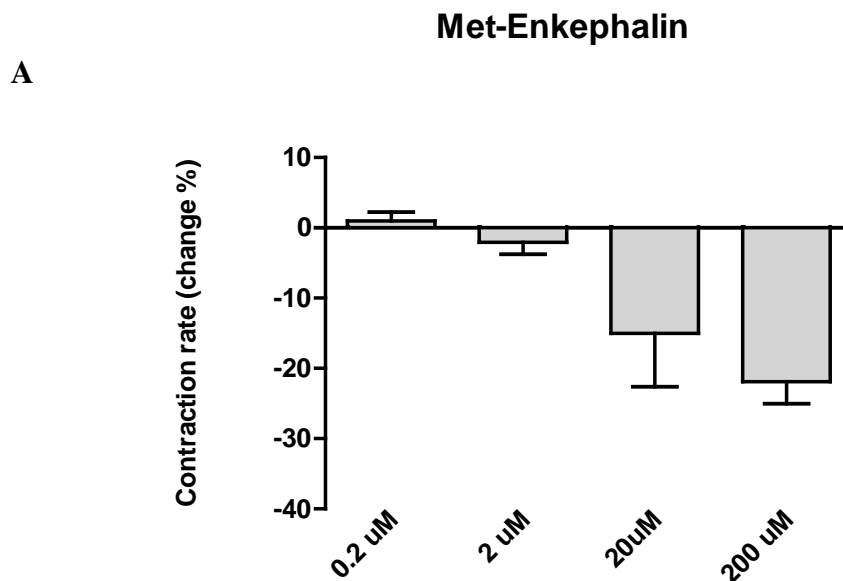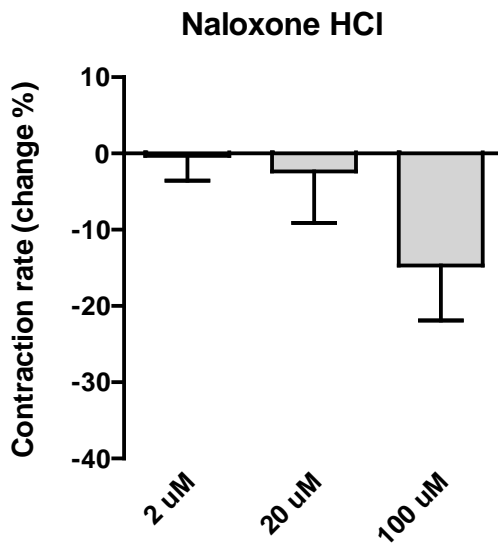
Figure 3.3 *Effects of pertussis toxin (PTX) pretreatment on negative chronotropic responses of berberine in neonatal murine cardiomyocyte.* Cultured neonatal cardiomyocytes were pretreated with PTX (100 ng/ml for 24h), then 2 µM carbachol (CCh) or 20 µM berberine (BB) was added to the cardiomyocytes. Contraction frequency was determined before and after the addition of berberine at the concentrations indicated. Chronotropic response is shown as a percentage of change in contraction rate, calculated as the difference between observed contraction rates in beats/min prior to and after treatment and related to the original contraction frequency for the observed cell. Means + S.E.M are shown. * indicates significant difference at $p < 0.05$ in average values as compared to control (PBS). # indicates significant difference at $p < 0.05$ between treatment groups in the absence and presence of PTX.

Different receptors in the heart such as opioid receptors, adenosine receptors, and muscarinic receptors are coupled by a G-protein (Gi/o) to adenylyl cyclase in an inhibitory fashion. Stimulation of these receptors causes a direct negative chronotropic effect. Therefore, we investigated whether berberine decreases contraction rate of cardiomyocytes via opioid receptors.

In this study, we determined the dose response effects for opioid agonist and antagonist on contraction rate of neonatal murine cardiomyocytes (Figure 3.4A, 3.4B). Met-enkephalin displayed a dose-dependent inhibitory effect on cardiomyocyte contraction rate reaching a maximum at approximately 20 µM. Pretreatment of cardiomyocytes for 2 min with 2 µM naloxone (opioid antagonist) did not block the negative chronotropic effect of berberine but did block the effect of 20 µM met-enkephalin (Figure 3.4C).

**Met-Enkephalin**
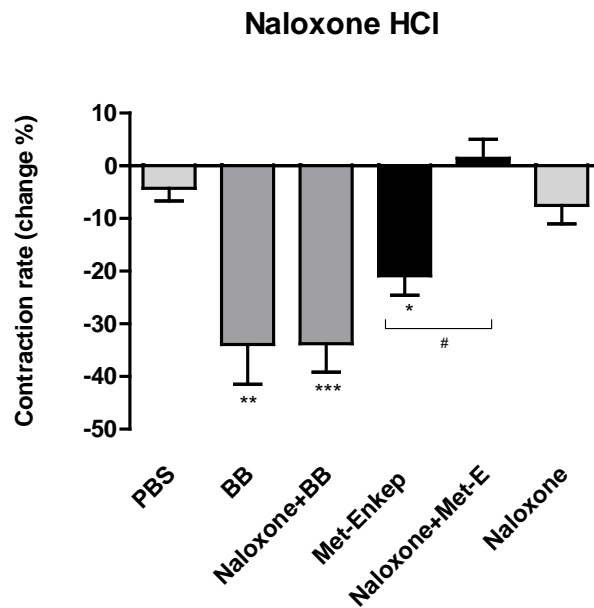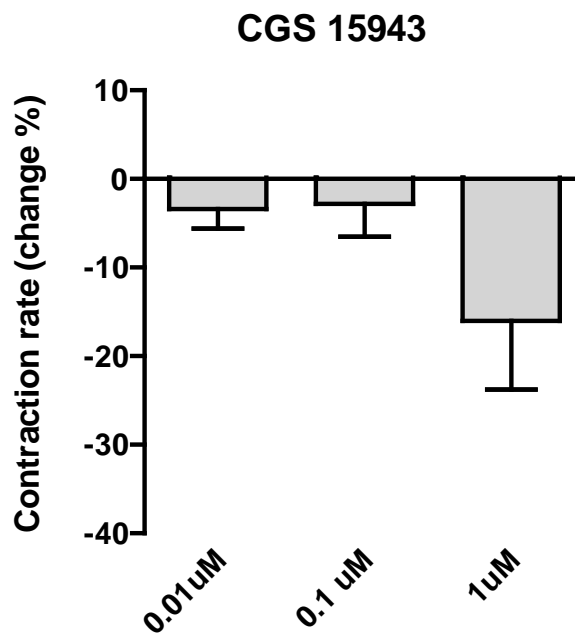
A

**B**



**C**



Figure 3.4 A, B) *Dose-response effects of opioid agonist (met-enkephalin) and antagonist (naloxone) on the spontaneous contraction rate of cultured*

*cardiomyocytes.* The negative chronotropic effect of drugs on cardiomyocytes was calculated as the difference between observed contraction rates in beats/min before and after treatment as a percentage of the original contraction frequency. Means $\pm$ S.E.M. are shown. C) *Effect of naloxone pretreatment on direct negative chronotropic response to berberine in neonatal murine cardiomyocytes.* Cultured neonatal cardiomyocytes were treated with PBS, 20µM berberine (BB), 2 µM naloxone or 20 µM met-enkephalin. Cardiomyocytes were pretreated for 2 min with 2 µM naloxone followed by 20 µM berberine (Naloxone+BB). After pretreatment, the contraction rate was determined before and after the addition of berberine at the concentrations indicated. Chronotropic response is shown as a percentage of change in contraction rate that was calculated as the difference between observed contraction rates in beats/min prior to and after treatment, and considered as a percentage of the original contraction frequency for the observed cell. Means + S.E.M are shown. *, **, and *** indicate significant difference at $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively in average values as compared to control (PBS). # indicates significant difference at $p < 0.05$ between treatment groups in the absence and presence of naloxone.

We determined the dose response effects for adenosine agonist (2-chloroadenosine) and antagonist (CGS 15943) on contraction rate of neonatal murine cardiomyocytes (Figure 3.5A, 3.5B respectively). As expected, 2-chloroadenosine displayed a dose-dependent inhibitory effect on cardiomyocyte contraction rate. Pretreatment of cardiomyocytes for 2 min with 0.1 µM CGS15943 (adenosine antagonist) did not block the negative chronotropic effect of berberine but did block the effect of 20 µM 2-chloroadenosine (Figure 3.5C).

A

**2-Chloroadenosine**
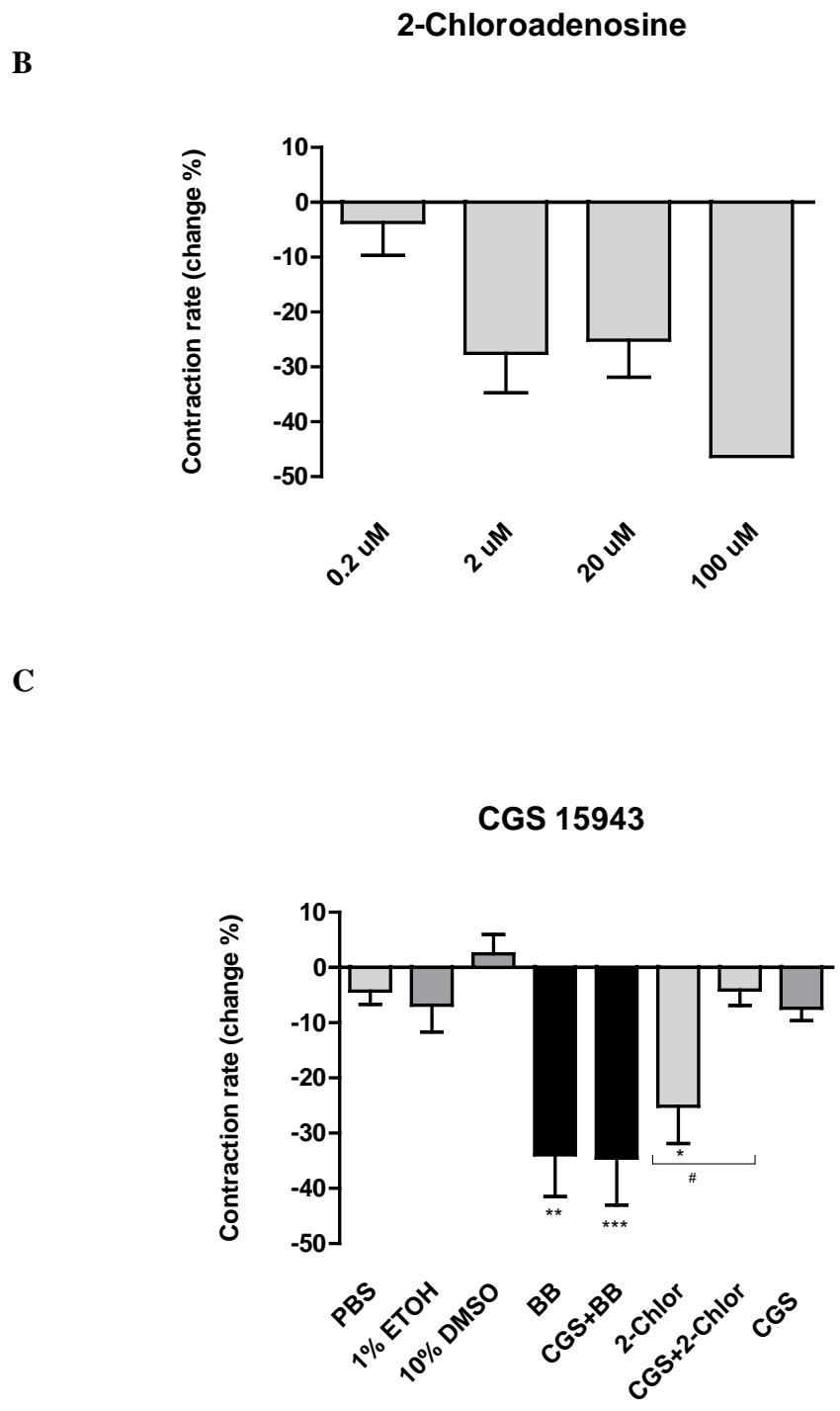
**B**



**C**

**CGS 15943**



Figure 3.5 A, B) *Dose-response effects of adenosine agonist 2-chloroadenosine, and antagonist CGS 15943 on the spontaneous contraction rate of cultured cardiomyocytes.* The negative chronotropic effect of drugs on cardiomyocytes was

calculated as the difference between observed contraction rates in beats/min before and after treatment as a percentage of the original contraction frequency. Means ± S.E.M. are shown. C) *Effect of CGS 15943 pretreatment on direct negative chronotropic response to berberine in neonatal murine cardiomyocytes*. Cultured neonatal cardiomyocytes were treated with PBS, 20µM berberine (BB), 0.1 µM CGS 15943 (CGS), or 20 µM 2-chloroadenosine (2-Chlor). Cardiomyocytes were pretreated for 2 min with 0.1 µM CGS 15943 followed by 20 µM berberine (CGS+BB).   After pretreatment, the contraction rate was determined before and after the addition of berberine at the concentrations indicated. Chronotropic response is shown as a percentage of change in contraction rate that was calculated as the difference between observed contraction rates in beats/min prior to and after treatment, and considered as a percentage of the original contraction frequency for the observed cell. Means + S.E.M are shown. *, **, and *** indicate significant difference at $p<0.05$, $p<0.01$, and $p<0.001$ respectively in average values as compared to control (PBS). # indicates significant difference at $p<0.05$ between treatment groups in the absence and presence of CGS 15943.

To examine whether berberine decreases the contraction rate of cardiomyocytes via $\alpha_2$-adrenoceptors, we investigated the effect of phentolamine on the negative chronotropic response of cardiomyocytes to berberine. First, we determined the dose response effects for clonidine and phentolamine on contraction rate of neonatal murine cardiomyocytes (Figure 3.6A, 3.6B respectively).

Pretreatment of cells for 2 min with 20 nM phentolamine had no significant effect on negative chronotropic activity of 20 µM berberine (Figure 3.6C).
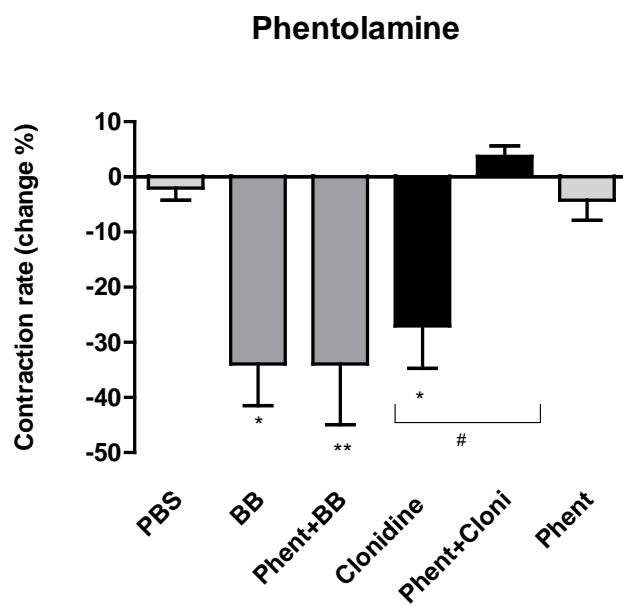
**A**
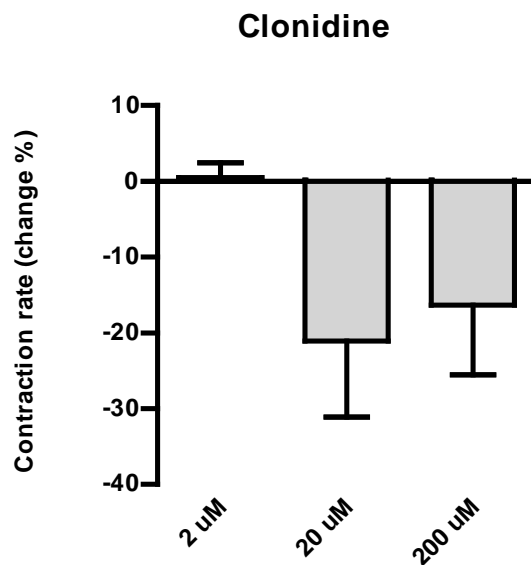
**B**
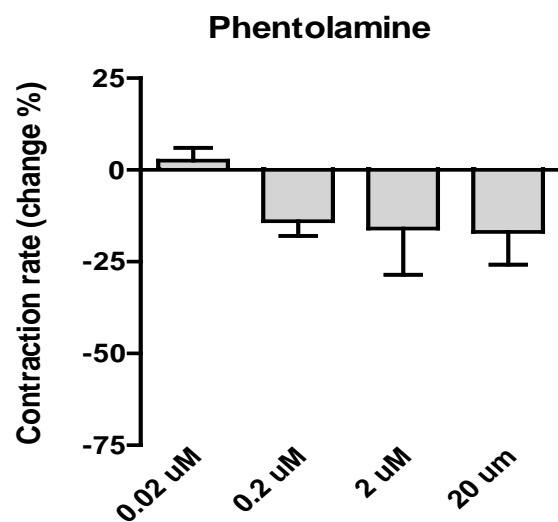


**C**



Figure 3.6 A, B) *Dose-response effects of clonidine and phentolamine on the spontaneous contraction rate of cultured cardiomyocytes.* The negative chronotropic
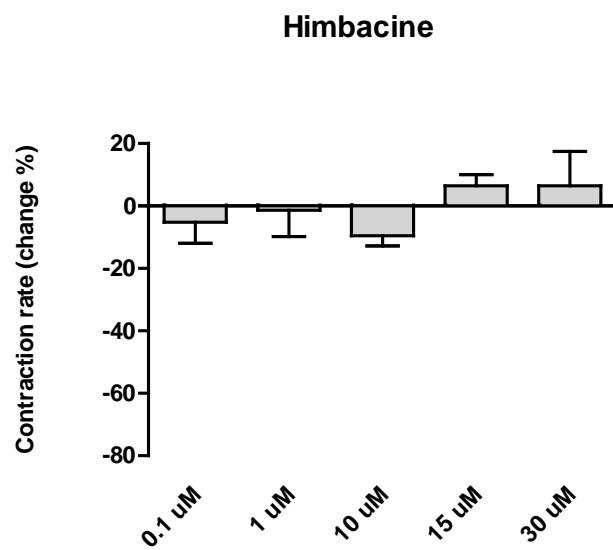
effect of drugs on cardiomyocytes was calculated as the difference between observed contraction rates in beats/min before and after treatment as a percentage of the original contraction frequency. Means ± S.E.M. are shown. C) *Effect of phentolamine pretreatment on direct negative chronotropic response to berberine in neonatal murine cardiomyocytes*. Cultured neonatal cardiomyocytes were treated with PBS, 20µM berberine (BB), 20 nM phentolamine (Phant) or 20 µM clonidine (Clon). Cardiomyocytes were pretreated for 2 min with 20nM phentolamine followed by 20 µM berberine (phentolamine+BB). After pretreatment, the contraction rate was determined before and after the addition of berberine at the concentrations indicated. Chronotropic response is shown as a percentage of change in contraction rate that was calculated as the difference between observed contraction rates in beats/min prior to and after treatment, and considered as a percentage of the original contraction frequency for the observed cell. Means + S.E.M are shown. * and ** indicate significant difference at $p<0.05$ and $p<0.01$ respectively in average values as compared to control (PBS).

   The similarity of berberine's effect to that of a muscarinic agonist was investigated by determining the effect of muscarinic antagonists on the chronotropic response of cardiomyocytes to berberine. First, we determined the dose response effects for himbacine, AF-DX 116 (M2-selective antagonists), and atropine on contraction rate of neonatal murine cardiomyocytes (Figure 3.7A, 3.7B, and 3.7C respectively).

   Atropine (0.1 mM) had no significant effect on cardiomyocyte contraction rate compared to vehicle, as expected.  However, pretreatment of cardiomyocytes for 2 min with 0.1 mM, 2.5 µM, and 0.1 µM atropine completely attenuated the negative chronotropic effect of berberine (Figure 3.7D, 3.7E, and 3.7F). The reported $K_D$ of atropine for M2 muscarinic receptors is 0.4 nM (Rashid et al., 2003).

   To further verify the specificity of atropine blockade on berberine's negative chronotropic activity, we also pretreated the neonatal cardiomyocyte preparations for 2 min with 0.01 mM himbacine or 0.5 µM AF-DX 116, selective M2-receptor antagonists. Berberine produced no significant negative chronotropic activity compared to PBS control in himbacine or AF-DX 116 pre-treated cardiomyocytes (Figure 3.6G, and 3.6H).

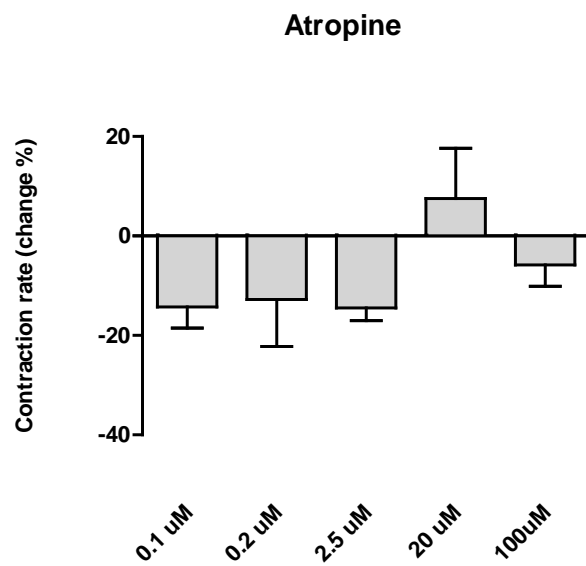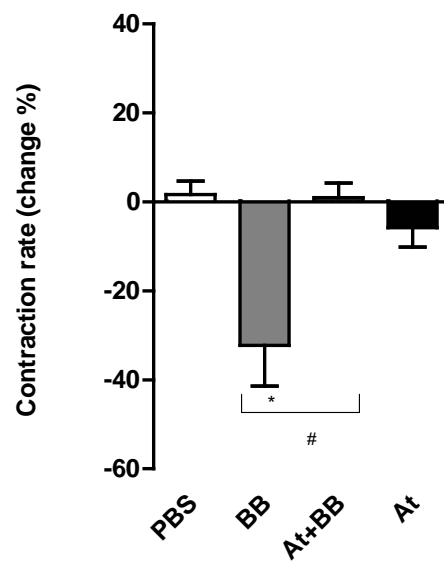**A**
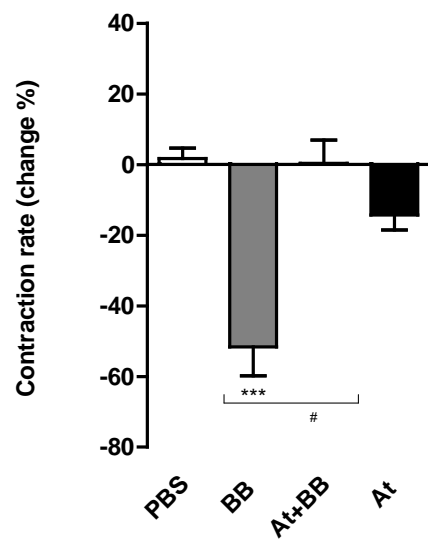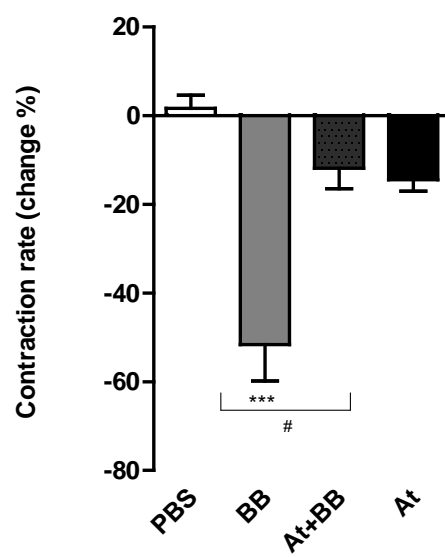
**Himbacine**



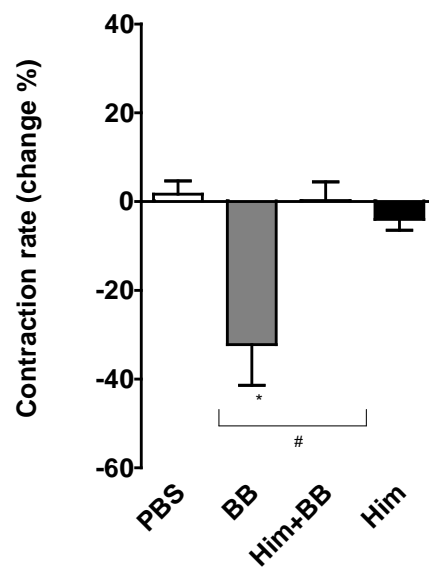**B**
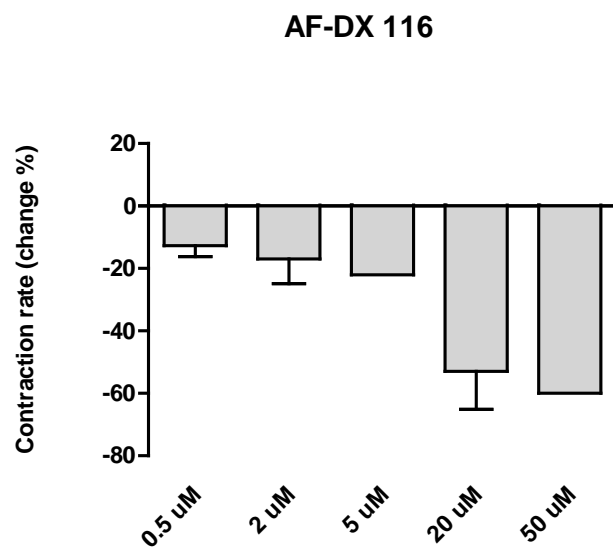
**AF-DX 116**

**C**



**D**

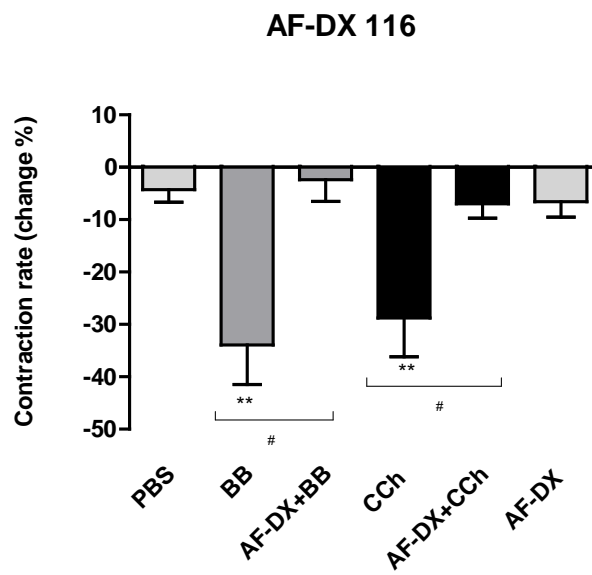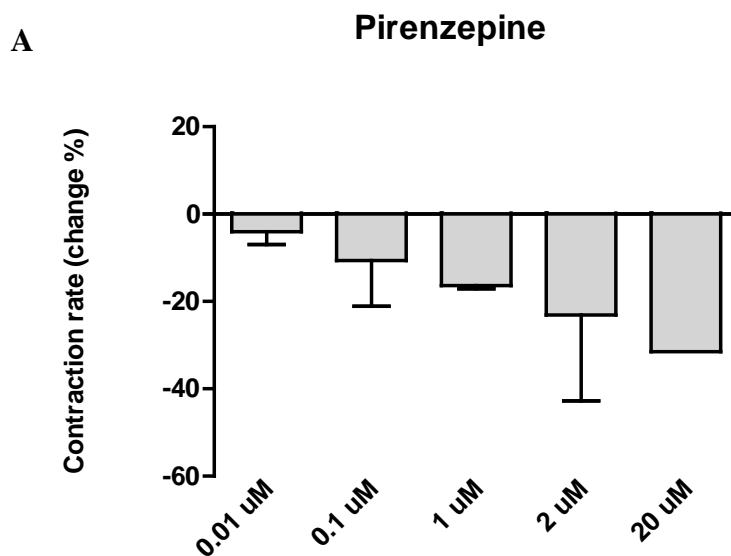**E**



**F**

**G**



**H**



Figure 3.7 A, B, and C) *Dose-response effects of himbacine, atropine, and AF-DX 116 on the spontaneous contraction rate of cultured cardiomyocytes.* The

chronotropic effect of drugs on cardiomyocytes was calculated as the difference between observed contraction rates in beats/min before and after treatment as a percentage of the original contraction frequency. Means ± S.E.M. are shown. D, E, F, G, and H) *Effect of muscarinic antagonist pretreatment on direct negative chronotropic response to berberine in neonatal murine cardiomyocytes.* Cultured neonatal cardiomyocytes were treated with PBS, or 20µM berberine (BB), 0.5 µM AF-DX 116 (AF-DX), 0.01 mM himbacine (Him), or different concentration of atropine (At) (0.1 µM, 2.5 µM, or 0.1mM). D) Cardiomyocytes pretreated for 2 min with 0.1 mM atropine (At) followed by 20 µM berberine (At+BB). E) Cardiomyocytes pretreated for 2 min with 0.1 µM atropine (At) followed by 100 µM berberine (At+BB). F) Cardiomyocytes pretreated for 2 min with 2.5 µM atropine (At) followed by 100 µM berberine (At+BB). G) Cardiomyocytes were pretreated for 2 min with 0.01 mM himbacine (Him) follows by 20 µM berberine (Him+BB). H) Cardiomyocytes were pretreated for 2 min with 0.5 µM AF-DX 116 (AF-DX) followed by 20 µM berberine (AF-DX+BB). After pretreatment, the contraction rate was determined before and after the addition of berberine at the concentrations indicated. Chronotropic response is shown as a percentage of change in contraction rate that was calculated as the difference between observed contraction rates in beats/min prior to and after treatment, and considered as a percentage of the original contraction frequency for the observed cell. Means + S.E.M are shown. *, **, and *** indicate significant difference at $p<0.05$, $p<0.01$, and $p<0.001$ respectively, in average values as compared to control (PBS). # indicates significant difference at $p<0.05$ between treatment groups in the absence and presence of atropine, himbacine, or AF-DX 116.

To further verify the specificity of M2- antagonists on berberine's negative chronotropic activity, we also pretreated the neonatal cardiomyocyte preparations with pirenzepine, a partially M1-selective receptor antagonist. The dose response to pirenzepine on cardiomyocyte contractility is shown in Figure 3.8A. In contrast to himbacine and AF-DX 116 (selective M2 antagonists), 0.02 µM pirenzepine, did not significantly affect negative chronotropic activity of berberine (Figure 3.8B). However, reflective of the partial M1 selectivity of pirenzepine, there was a non significant tendency toward reduction of carbachol and berberine's effects.

**A**

**Pirenzepine**

**B**
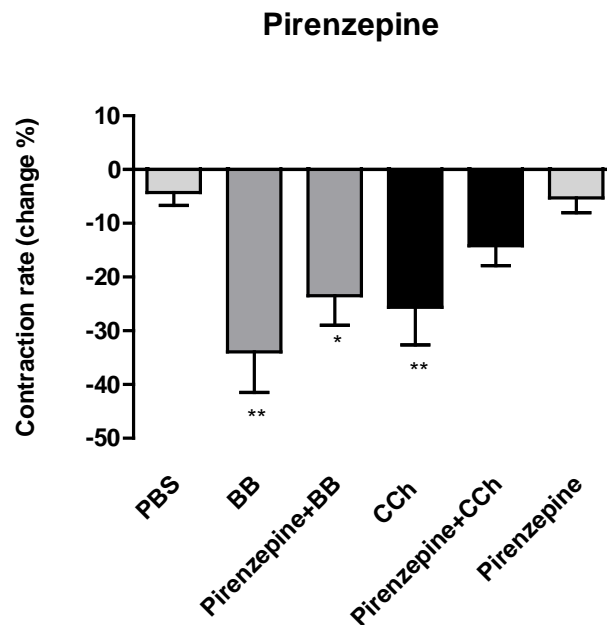


Figure 3.8 A) *Dose-response effects of pirenzepine on the spontaneous contraction rate of cultured cardiomyocytes.* The negative chronotropic effect of drug on cardiomyocytes was calculated as the difference between observed contraction rates in beats/min before and after treatment as a percentage of the original contraction frequency. Means ± S.E.M. are shown. B) *Effect of pirenzepine pretreatment on direct negative chronotropic response to berberine in neonatal murine cardiomyocytes.* Cultured neonatal cardiomyocytes were treated with PBS, 20µM berberine (BB), 20 nM pirenzepine, 2 µM carbachol (CCh). Cardiomyocytes were pretreated for 2 min with 20 nM pirenzepine followed by 20 µM berberine (pirenzepine+BB). After pretreatment, the contraction rate was determined before and after the addition of berberine at the concentrations indicated. Chronotropic response is shown as a percentage of change in contraction rate that was calculated as the difference between observed contraction rates in beats/min prior to and after treatment, and considered as a percentage of the original contraction frequency for the observed cell. Means + S.E.M are shown. * indicates significant difference at $p < 0.05$ in average values as compared to control (PBS).

Binding affinity

A direct interaction of berberine with muscarinic receptors was investigated using a competitive radioligand binding assay in adult mouse heart membranes. $K_D$ and $B_{max}$ values for [³H]QNB binding are the same in membranes from adult and neonatal murine heart (Evans et al., 1985). Radiolabeled muscarinic antagonist [³H]QNB bound to a saturable single site with a $B_{max}$ value of $71.4 \pm 4.4$ fmol/mg and a $K_D$ value of $0.32 \pm 0.07$ nM in our mouse heart membranes preparation. Berberine was evaluated for ability to displace 0.4 nM [³H]QNB binding in mouse heart membranes. Competition binding experiments demonstrated that berberine displaced [³H]QNB binding in a dose dependent manner (Figure 3.9A) with $K_i = 2.4 \times 10^{-6}$ M.

Assuming that the bradycardia produced by berberine was mediated by activation of muscarinic M2 receptors, we sought assay of the affinity of berberine specifically at M2 receptors in a cell line devoid of other cholinergic receptor subtypes. Porcine muscarinic M2 receptors were transiently over-expressed in HEK 293 cells. The binding curve of [³H]QNB to M2 receptor-transfected HEK 293 membranes was best fit to a saturable single site with a $B_{max}$ value of $3804 \pm 390$ fmol/mg and a $K_D$ value of $1.56 \pm 0.30$ nM. In non-transfected HEK 293 cells, we were not able to measure specific [³H]QNB binding (data not shown). Competition binding experiments demonstrated that berberine displaced [³H]QNB binding to M2 receptors of transfected HEK 293 membranes with $K_i = 4.9 \times 10^{-6}$ M (Figure 3.9B).

**A**



**B**
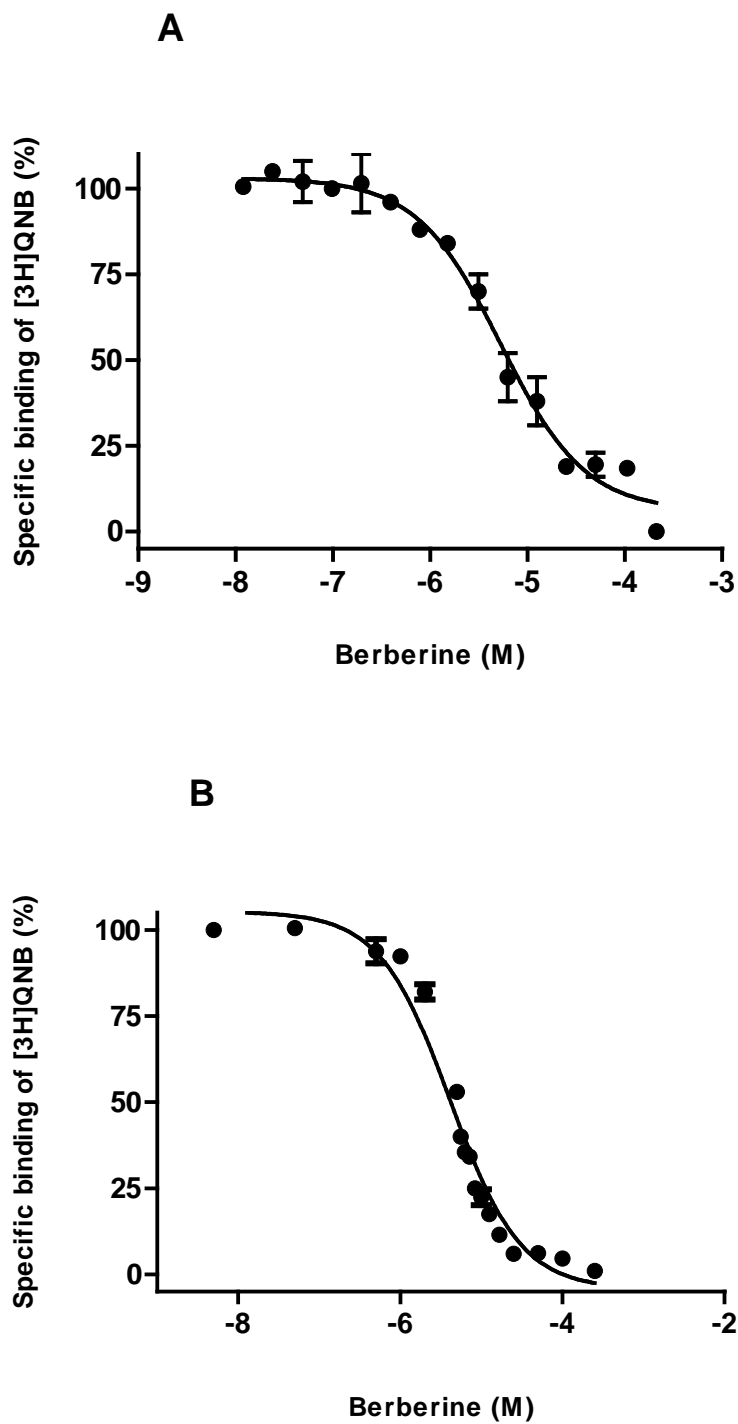


Figure 3.9 *Competition binding profile of berberine against [³H]QNB in mouse heart membranes*. Indicated concentrations of berberine were co-incubated with 0.4

nM [³H]QNB in radioligand competition binding assays with **A**) adult heart mouse heart membrane or **B**) M2-transfected HEK cell membranes. Radioactivity retained by filters is quantitated and nonspecific binding in the presence of 0.1 mM atropine is subtracted from all data points. Data represent the mean ± S.E.M. of more than three determinations, each performed in triplicate and expressed as the percentage of specific binding. $K_i = 2.4 \times 10^{-6}$ M for mouse heart membranes and $K_i = 4.9 \times 10^{-6}$ M for M2-transfected HEK cell membranes.

Inhibition of $[^3H]$QNB binding by carbachol and berberine in adult cardiac and transfected HEK 293 homogenates is summarized in table 3.1. For both carbachol and berberine, $EC_{50}$ values for decreasing contraction rate were approximately one order of magnitude lower than $K_i$ values, suggesting the presence of spare receptors in both systems for both agonists.

## Discussion

The main findings of this study are: (a) in neonatal murine cardiomyocytes, berberine has a significant negative chronotropic effect, (b) berberine does not possess adrenoceptor blocking action, (c) PTX pretreatment significantly reduced the negative chronotropic effect of berberine, (d) atropine, himbacine, or AF-DX 116 muscarinic receptor antagonists blocked the direct negative chronotropic activity of berberine, (e) competition binding studies showed that berberine clearly bound to muscarinic receptors in mouse heart and M2 receptor-transfected HEK cell membranes with micromolar affinity, similarly to the classic agonist, carbachol. In summary, these data support our hypothesis that berberine decreased the contraction rate of neonatal mouse cardiomyocytes via muscarinic receptor activation. To our knowledge, this is the first report to show that berberine has high affinity to M2 muscarinic receptors.

The negative chronotropic effect of berberine observed here is consistent with the findings of Shaffer (1985), and Riccioppo Neto (1993), who have reported a bradycardic effect of berberine in guinea pig and rabbit hearts. On the other hand, Sabir and Bhide (1971) showed a 10-30% increase in contraction rate of isolated right atria of rabbits. According to Shaffer's explanations, there are some reasons for the

differences between the results of Sabir's group and others. For example, they used different species, different temperature, and different bathing solutions (Shaffer, 1985). Activation of cardiac muscarinic receptors producing dual and opposite contraction-dependent effects on the heartbeat has been described in almost all cardiac cell types in virtually every species. Application of low concentrations of carbachol produced an atropine-sensitive decrease in spontaneous contraction rate of cultured neonatal rat ventricular myocytes while higher concentrations of carbachol, under appropriate conditions, elicits stimulatory effects on the contraction rate (Colecraft et al., 1998).

In the cardiomyocytes of this study, the chronotropic activity of berberine was not inhibited by naloxone, an opioid receptor antagonist, CGS 15943, an adenosine receptor antagonist, or a α-adrenoceptor antagonist, phentolamine. Therefore, berberine may not decrease the contraction rate of cardiomyocytes via opioid, adenosine, or α receptors.

It is unlikely that blockade of adrenergic receptors is responsible for the negative chronotropic activity that was produced by berberine in cardiomyocytes. First, there is no neuronal source of cardiac stimulant in the cultures. Second, Riccioppo Neto (1993) demonstrated in isolated right atria of rabbits that berberine did not alter the positive chronotropic effect of isoproterenol. Third, berberine did not affect the ability of epinephrine to increase contraction rate in our cultures.

In our study, the bradycardic effect of berberine was blocked completely by atropine, a non-selective muscarinic antagonist. Thus, chronotropic effect of berberine in neonatal murine cardiomyocytes is mediated by a muscarinic receptor.

The M1-receptor preferential antagonist, pirenzepine, did not significantly block the bradycardic effect of berberine.

Muscarinic receptors in the heart of various mammalian species are predominantly of the M2-subtype which transduces signals via a PTX-sensitive G-protein (Gi/o). In the present study, we observed that PTX pretreatment attenuated the bradycardic effect of berberine consistent with activation of a muscarinic (M2-type) receptor.

Further, himbacine or AF-DX 116, more selective antagonists of M2 muscarinic receptors, also completely abolished the negative chronotropic effect of berberine. Our data contradict the findings of Shaffer (1985) and Riccioppo Neto (1993) in isolated guinea pig atria or rabbit sinoatrial cells respectively, where atropine did not prevent berberine-induced bradycardia. The reason for the discrepancy between our data and those published by Shaffer and/or Riccioppo Neto is not known. However, we have examined the exact doses of berberine (100 µM) and atropine (0.1 µM and 2.5 µM) which Shaffer and Riccioppo Neto, respectively, used in their experiments. Our results showed that atropine at those concentrations still blocked the negative chronotropic effect of berberine in cultured neonatal murine cardiomyocytes in our hands. Perhaps a reason for the discrepancy relates to the concentrations used and the different experimental systems. Shaffer and Riccioppo Neto used five times more berberine and  upto 2400 times lower atropine in their assays, so perhaps they are too close to the edge of atropine being effective in a system where it must penetrate the tissue. Morever, neither group published data using a control such as carbachol to

show that atropine at the concentrations used was an effective antagonist in their system.

In addition, we demonstrated that berberine dose-dependently inhibited [$^3$H]QNB binding to M2 muscarinic receptor of transfected HEK 293 membranes. Preeviously, Schmeller and co-workers (Schmeller et al., 1997) convincingly demonstrated that in porcine brain, berberine clearly binds to muscarinic receptors with an $IC_{50}$ of 1 µM. These data are in agreement with our binding data in heart cell membranes. Taken together, our findings suggest that the negative chronotropic activity of berberine in neonatal murine cardiomyocyte may be mediated via muscarinic receptor activation.

# Chapter 3:  Effect of berberine on hypertrophy of H9c2 cells

Satin Salehi and Theresa M. Filtz

In progress

## Abstract

Berberine is the basic chemical component of a Chinese herb, *Coptis chinensis* Franch (coptis), considered to be useful in treating some diseases of the cardiovascular system such as hypertension and heart failure. Congestive heart failure is a common clinical syndrome characterized by abnormalities of cardiac function and morphology.

Cardiac hypertrophy represents the most important factor in the development of congestive heart failure. In this study, we investigated the inhibitory effect of berberine on hypertrophy of H9c2 cells. In rat heart-derived H9c2 myoblast cells treated with different hypertrophic agonists such as insulin growth factor II (IGF-II, 20 nM), arginine vasopressin (AVP, 2 μM), phenylephrine (α-agonist, 20 μM), and isoproterenol (β-agonist, 20 μM ), for 24 or 48 hours, protein content and size of cells were significantly increased compared to control group. However, H9c2 cellproliferation was not affected by hypertrophic agonists compared to control. The increases in cell area and protein content induced by the hypertrophic agonists were inhibited by treatment with berberine in a concentration-dependent manner. Our findings have provided the first scientific evidence that berberine may have an inhibitory effect on hypertrophy of heart-derived cells, and may provide a rationale for further studies to evaluate berberine's cardiac activity, or suggest a molecular scaffold from which to build other molecules with anti-hypertrophic properties.

## Introduction

Cardiac hypertrophy is considered a major cause of heart disease such as heart failure. Cardiac function is compromised in various stressful situations such as hypertension, myocardial infarction, or exercise; the heart attempts to maintain normal function by proceeding to cellular hypertrophy (Kerkela and Force, 2006). The defining features of hypertrophy are an increase in cardiomyocyte size, enhanced protein synthesis, and induction of a hypertrophic gene program increasing factors such as ANP. Prolonged hypertrophy is related to cardiomyocyte appotosis and a significant increase in the risk for sudden death or congestive heart failure (Frey and Olson., 2003; Schluter et al., 1999; Hong et al., 2003).

Cardiomyocytes of adult myocardium increase their cellular mass in response to growth stimuli such as classical growth hormones and various neuroendocrine factors (Schluter et al., 1999). Much evidence demonstrates that insulin growth factor II (IGF-II), arginine vasopressin (AVP), angiotensin and adrenergic agonist (e.g. phenylephrine α-agonist, and isoproterenol β-agonist) are involved in or are stimuli for myocyte hypertrophy (Frey and Olson., 2003).

Several studies reported that [Arg$^8$]-vasopressin (AVP) increases the rate of protein synthesis in cardiomyoctes and in heart in neonatal mice, rats and also in humans, indicating that AVP might induce pathological hypertrophy. AVP increased the expression of ANP mRNA and protein in mouse neonatal cardiomyocytes. The hypertrophic effects of AVP were significantly inhibited by a selective vasopressin

$V_{1A}$ receptor antagonist, OPC-21268, suggesting that AVP promotes cardiomyocyte hypertrophy via the vasopressin $V_{1A}$ receptor (Hiroyama et al., 2007).

H9c2 cells express $V_1$ receptors and respond to vasopressin with an increase in release of $Ca^{2+}$ from sarcoplasmic reticular S(E)R $Ca^{2+}$ stores, increase of intracellular $Ca^{2+}$, and finally activation of phospholipase C-β, protein kinase C (PKC) and the p42 MAP kinase (ERK2) (Brostrom et al., 2000).

H9c2 cells, a permanent cell line derived from rat cardiac tissue, show morphological characteristics similar to those of immature embryonic cardiomyocytes, but they also have several elements of the electrical and hormonal pathway found in adult cardiomyocytes, such as the L-type $Ca^{2+}$ channel, $K^+$ channels (ATP-sensitive $K^+$ channels and voltage-sensitive $K^+$ channels, Kv2.1, Kv3.4, Kv4.1, Kv4.3), and heterotrimeric G-proteins. Therefore, these cells are useful as a model for cardiomyocytes in transmembrane signal transduction studies (Hescheler et al., 1991). However, these cells do not contract.

In heart-derived H9c2 cells, hypertrophic agonists such as angiotensin II, phenylephrine, isoproterenol, and urotensin I reporetedly significantly increased cell size by 27-47% (Hwang et al., 2006; Huang et al., 2004). H9c2 cells treated for 24h with vasopressin had protein content 35% higher compared to control preparations, and the two preparations did not differ significantly with respect to cell number (Brostrom et al., 2000). Therefore, H9c2 myocytes provide a suitable model of cell hypertrophy via various cardiac hypertrophic agonists.

Berberine [5,6-dihydro-9,10-dimethoxy-benzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium], an alkaloid isolated from a variety of plants such as *Berberis*

*aquifolium* and *Berberis aristata*, has a long history in traditional Chinese medicine (Lau et al 2001; Cheng et al., 2006; Yang et al., 2006). Animal and clinical studies have suggested a number of beneficial effects of berberine on cardiovascular system. It is reported that berberine and its derivatives have positive inotropic and antiarrhythmic properties. Recently, the efficacy and safety of berberine was assessed for the treatment of chronic heart failure, and it was showed that berberine increased left ventricular ejection fraction and exercise capacity, improved quality of life and decreased mortality in patients with congestive heart failure (Lau et al., 2001; Yang et al., 2006; Zeng et al., 2003). In a previous study, it was shown that berberine could improve the damaged heart function of chronic heart failure rats caused by aortic banding and showed beneficial effects in inhibiting the development of cardiac hypertrophy (Hong et al., 2002). It was indicated that the inhibitory effect of berberine on cardiac hypertrophy is associated with modulation of sympathetic nerve activity. In experimental cardiac hypertrophy in rats Hong et al. reported that berberine decreased the level of noradrenaline and adrenaline in plasma (Hong et al., 2003).

In order to further explore the effect of berberine to potentially inhibit the development of cardiac hypertrophy, we investigated whether berberine directly blocks cellular hypertrophy in H9c2 cells treated with the hypertrophic agonists, insulin growth factor II (IGF-II, 20 nM), arginine vasopressin (AVP, 2 $\mu$M), phenylephrine ($\alpha$-agonist, 20 $\mu$M), and isoproterenol ($\beta$-agonist, 20 $\mu$M ). It has been accepted that AVP induced hypertrophy through $V_{1A}$ receptors; therefore in our study we used SR 49059, a selective $V_{1A}$ receptor antagonist, to block AVP action on

hypertrophy of H9c2 cells. Our findings indicate that berberine suppresses hypertrophy induced by hypertrophic agonists in H9c2 cells. The inhibitory effect of berberine may be developed to have some therapeutic potential in the treatment of CHF.

## Materials and Methods

### Cell culture

H9c2 cells were subcultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin). For cellular hypertrophy assays, cells were seeded in six-well plates at density of $15 \times 10^4$ and cultured for 24 h in DMEM containing 10% FBS. The cells were washed with serum-free medium (DMEM without serum) and then treated with potential hypertrophic agonists, insulin-growth factor II (20 nM), isoproterenol (20 μM), phenylephrine (20 μM), or arginine vasopressin (2 μM), with or without berberine. Cells were incubated for another 24 h at 37 °C in a humidified atmosphere containing 5% CO2 to induce hypertrophic responses. The cells were harvested for analysis.

### Protein determinations

H9c2 cells in 6-well plates were harvested by lysis with 1% sodium dodecyl sulfate for 30 min at 37º C followed by 3 min at 100º C. Protein content of aliquots of replicate lysates was determined by the Lowry method (Peterson et al., 1979; Lowry et al., 1951) using bovine serum albumin as standard.

## Cell area measurement

After washing cell monolayers with phosphate-buffered saline, images were obtained using a digital camera attached to a Leica inverted microscope (Leica, Chatsworth, CA, USA) with a 10x objective for analysis. Four random photographs were taken from each sample. Cell area was quantitated using Image J software on digitized photomicrographs

(Image Processing and analysis in Java; http://rsb.info.nih.gov/ij/index.html).

## Number of cell determinations

Live cells in 6-well plates were collected by trypsinization and counted on a hematocytometer with 6 replicates per treatment group (Hausser Scientific, Horsham, PA, USA).

## Data Analysis

All values are expressed as the mean $\pm$ S.E.M. with all experiments independently repeated a minimum of 3 times for averaging. Data were analyzed by one-way analysis of variance (ANOVA) followed Tukey's test for comparison of more than three groups. In all comparisons, the difference was considered to be statistically significant at $P < 0.05$.

## Materials

H9c2 cloned rat embryonic ventricular myocytes were obtained from the American Type Culture Collection. Arginine vasopressin was from Calbiochem, (Layolla, CA, USA). Insulin-growth factor II (IGF-II) and isoproterenol were purchased from Sigma-Aldrich, (St Louis, MO, USA). Phenylephrine was from TCI America (Portland, USA).  Trypsin, and Dulbecco's modified Eagle's medium were

purchased from Mediatec, Inc. (Herndon, VA, U.S.A.). Berberine sulfate was from Tokyo Kaser Kogyo Co. (Tokyo, Japan). SR49059 was purchased from Tocris biosciences (Ellisville, MO, USA).

## Results

### Effect of berberine on cell size of H9c2

We investigated whether the treatment with AVP would directly induce cell hypertrophy in H9c2 cardiomyoblast cells, and compared its effect with SR 49059 alone or in combination with SR 49059 (Figure 4.1). Exposure to 1 µM SR 49059 itself did not alter cell size of H9c2 cells compared to control. Pretreatment of cultured H9c2 with 1µM SR 49059 inhibited a 2 µM AVP-induced hypertrophy, as expected. The increase in cell size induced by AVP was not significantly inhibited by pretreatment with 10 µM of berberine although it clearly trended towards inhibiting AVP-induced increases in cell area.

In control H9c2 cells treated with insulin-growth factor II (20 nM), phenylephrine (20 µM), or isoproterenol (20 µM), cell size was significantly increased by 15-53% (Figure 4.2). The increase in cell size induced by all three hypertrophic agonists was significantly inhibited by pretreatment with 10 µM of berberine.
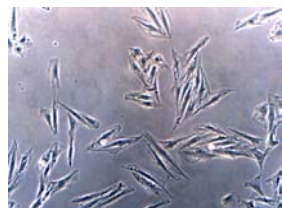
**A**



Cont

SR 49059

Cont

BB

AVP

SR 49059+AVP
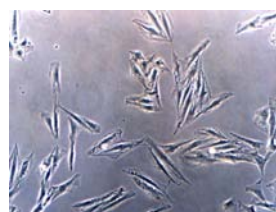
AVP
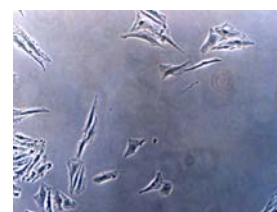
BB+AVP

B
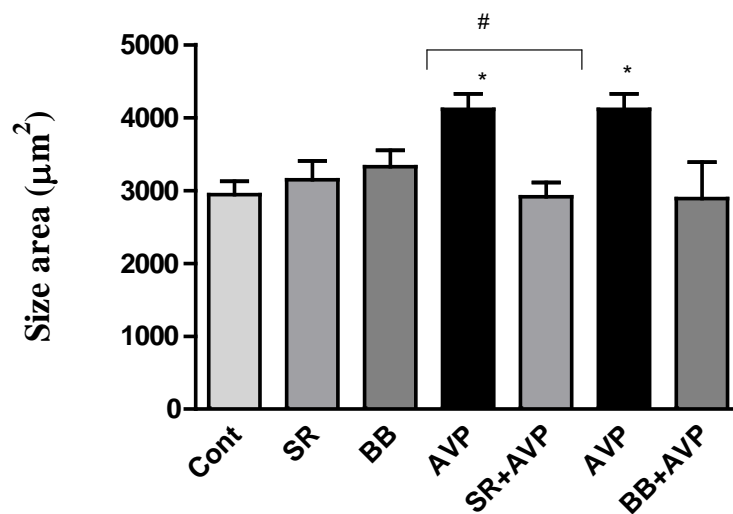


Figure 4.1 A) Morphological changes of H9c2 myocytes after treatment with 2 µM arginine vasopressin (AVP), 1µM SR 49059 (SR), or 10 µM berberine (BB). **B)** Effects of berberine and SR 49059 on hypertrophy induced by AVP in H9c2 cells. Cell area was quantitated using Image J software on digitized photomicrographs. Values represent the area + SEM. * indicates significant difference at $p<0.05$ in average values as compared to control group (no treatment). # indicates significant difference at $p<0.05$ between treatment groups in the absence and presence of berberine.

A



Cont            BB

IGF II            BB+IGF II

ISO            BB+ISO

PE            BB+PE
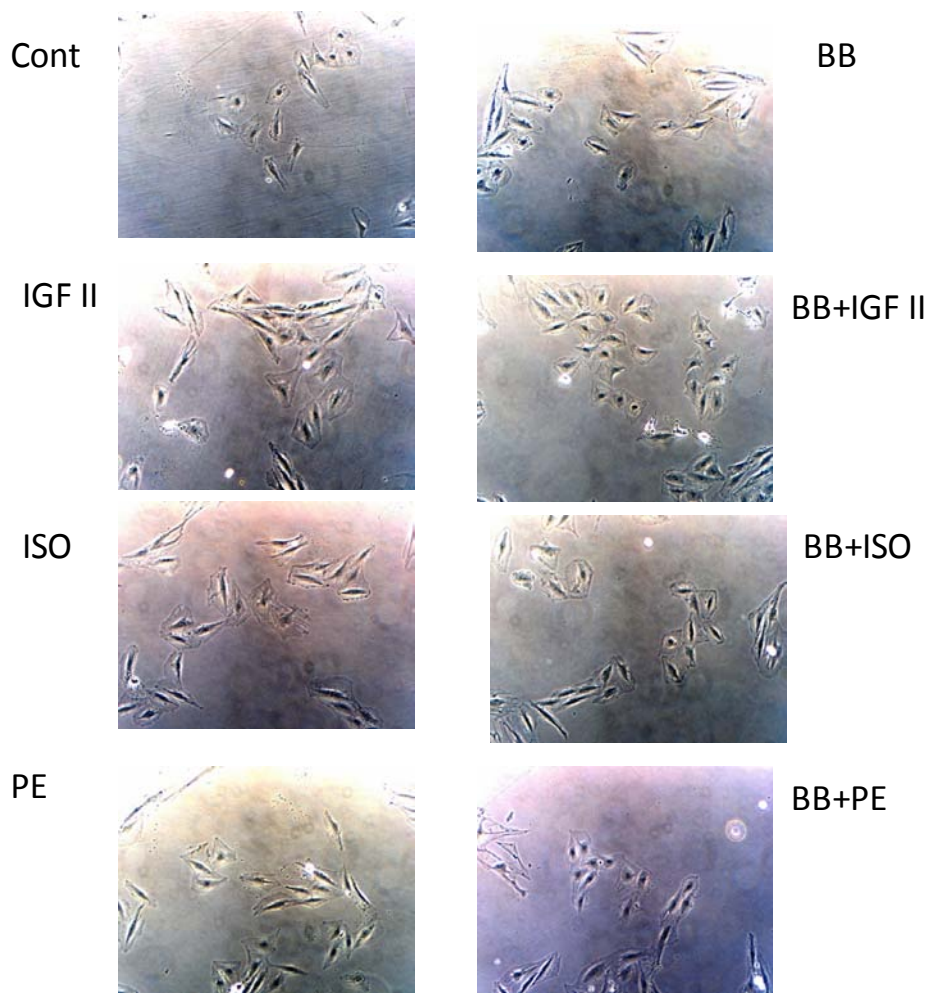
B
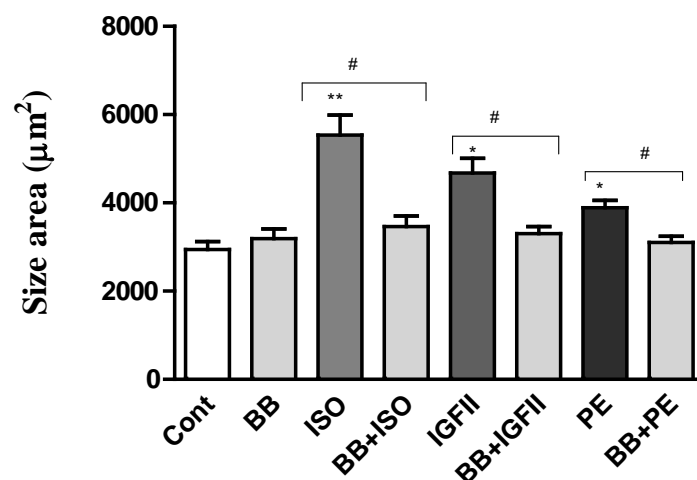


Figure 4.2 A) Morphological changes of H9c2 myocytes after treatment with the hypertrophic agonists 20 nM insulin-growth factor II (IGF II), 20 µM isoproterenol (ISO), 20 µM phenylephrine (PE) and the inhibitory effects of combination with 10 µM berberine (BB). H9c2 cells were pretreated for 30 min with 10 µM berberine and then 20 nM insulin-growth factor II (IGF II), 20 µM isoproterenol (ISO), or 20 µM phenylephrine (PE) was added to the cells. B) Effects of berberine on hypertrophy induced by IGF II, isoproterenol, or phenylephrine in H9c2 cells. Cell area was quantitated using Image J software on digitized photomicrographs. Values represent the area + SEM. *and ** indicate significant difference at $p<0.05$ and $p<0.01$ respectively in average values as compared to control group (no treatment). # indicates significant difference at $p<0.05$ between treatment groups in the absence and presence of berberine.
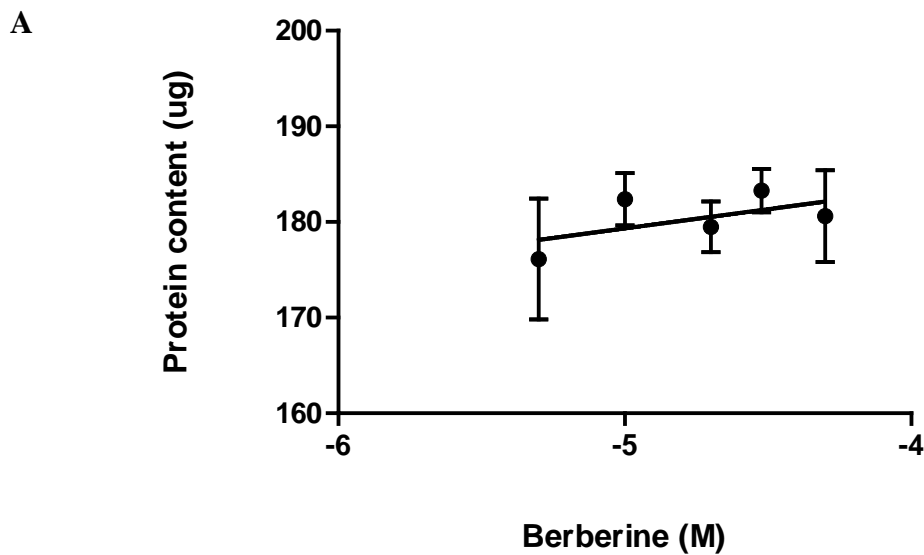
Effect of berberine on protein content of H9c2

In this study, we determined the dose response effects of berberine, SR 49059 ($V_{1A}$ selective antagonist) and hypertrophic agonists such as IGF II, AVP, isoproterenol, or phenylephrine on protein content of rat-derived H9c2 cells (Figure 4.3A to E). Exposure to 10 µM berberine or 1 µM SR 49059 itself did not significantly increase protein content of H9c2 cells compared to control (Figure 4.3A, 4.3B). In fact, at concentrations above 1 µM, SR 49059 appeared to shrink H9c2 cells, a marker of cell distress.

**A**

**B**
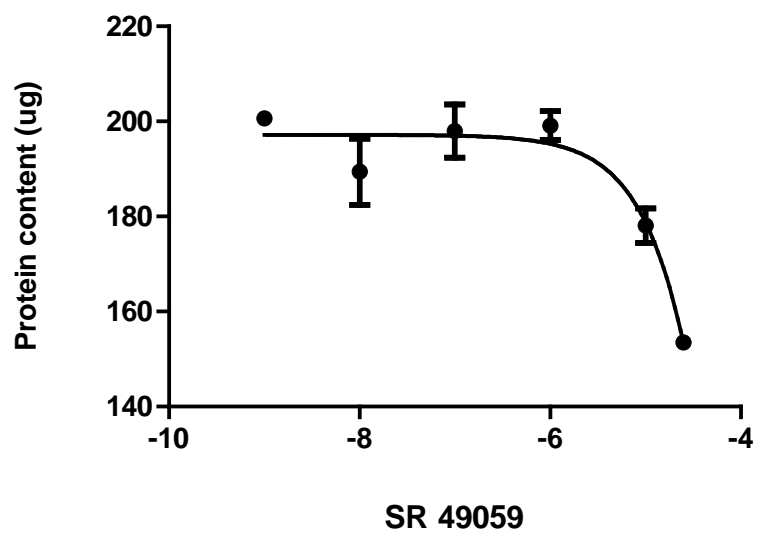


**C**
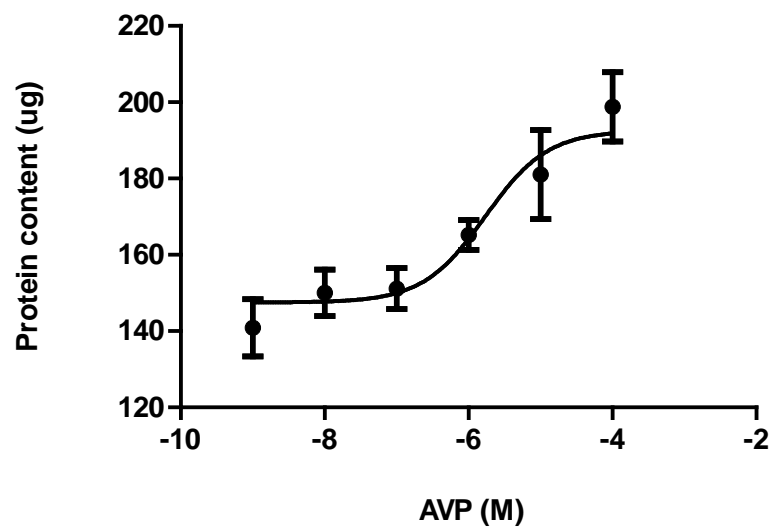
D
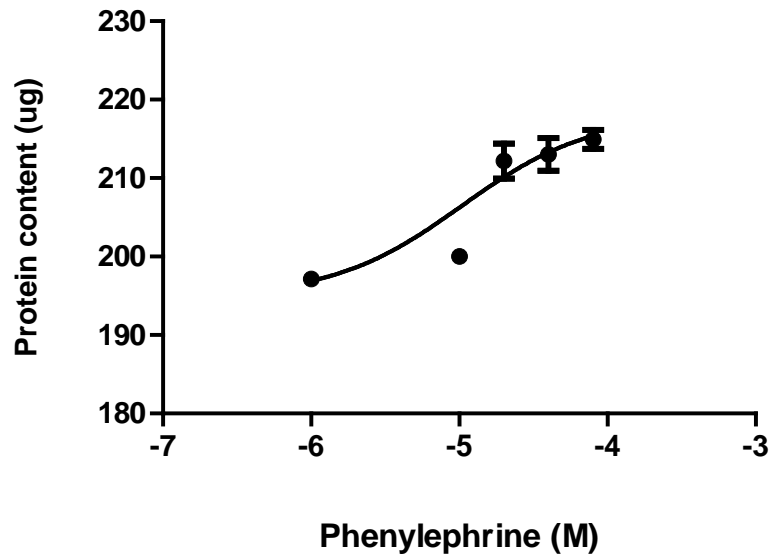


E
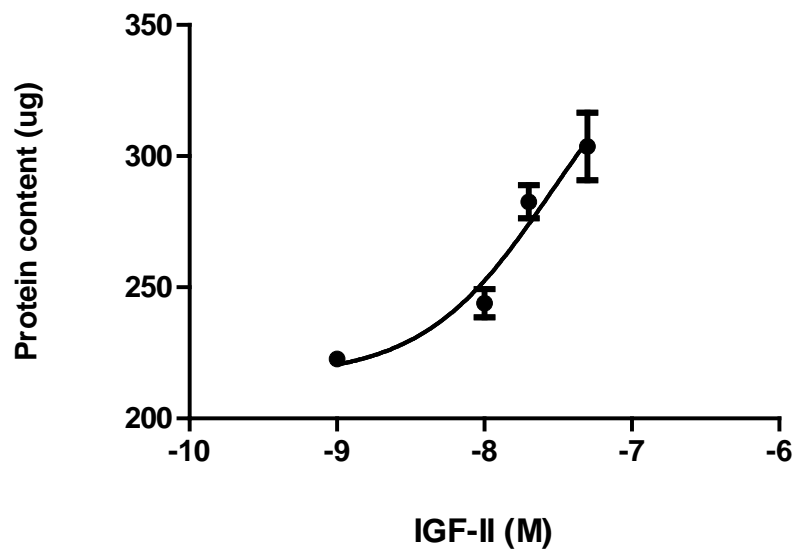


Figure 4.3 A to E) *Dose-response effects of berberine, SR 49059, and hypertrophic agonists such as AVP, IGF-II, and phenylephrine on protein content of H9c2 cells.* Cells were treated for 24h with various agents. Values are expressed as Means ± S.E.M at each dose. Protein concentrations were determined as described in methods.

In H9c2 cells treated with insulin-growth factor II, (IGF-II, 20 nM), arginine vasopressin (AVP, 2 µM), phenylephrine (PE, 20 µM), and isoproterenol (ISO, 20 µM), for 24 or 48 hours, protein content was significantly increased compared to control (Figures 4.4A -D). This data consistent with the cell area quantitation of hypertrophy.

The inhibitory effects of berberine on agonists-induced hypertrophy are presented in figures 4.4A-D, as measured by changes in protein content. Pretreatment of H9c2 cells with 10 µM berberine inhibited the hypertrophic agonist effects on protein content almost to the untreated control level for all agents tested; IGF-II (20 nM), AVP, (2 µM), phenylephrine (20 µM), and isoproterenol (20 µM ) in cultured H9c2 cells.
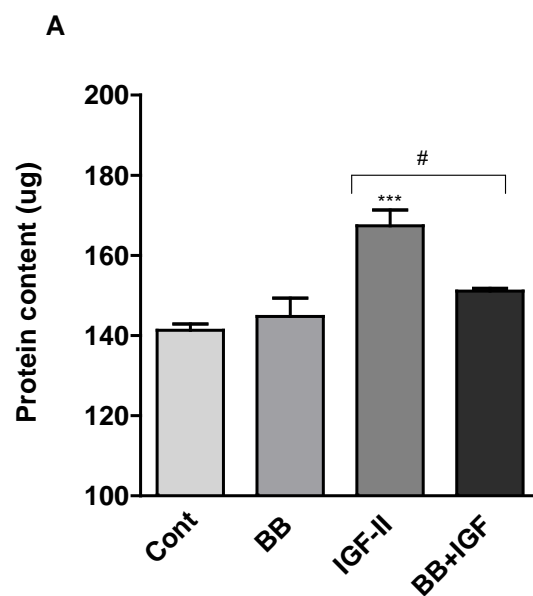
**B**
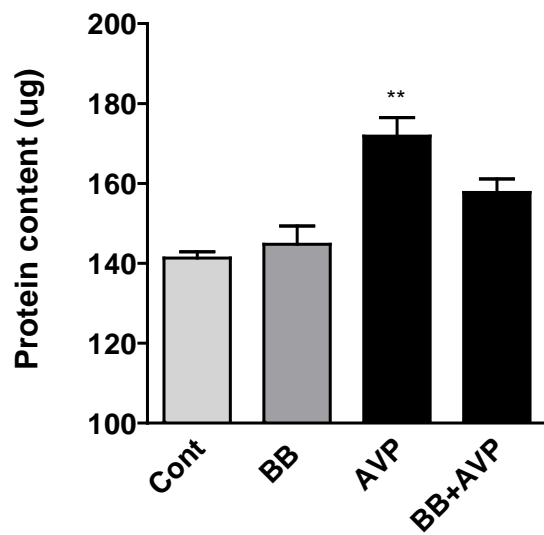


**C**
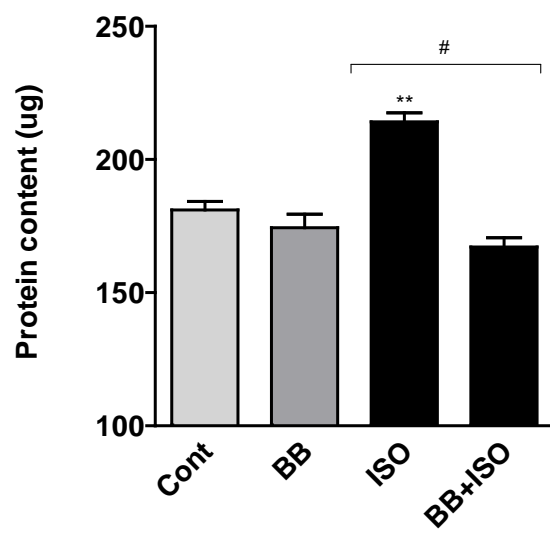
**D**



Figure 4.4A to D *Effect of hypertropic agonists on protein content of H9c2 cells pretreated with berberine.* A) Cultured H9c2 cells were treated with 20nM IGF II with (BB+IGF-II) or without 10µM berberine (BB).  B) Cultured H9c2 cells were treated with 2 µM arginine vasopressin (AVP) with (BB+AVP) or without 10 µM berberine (BB).  C) Cultured H9c2 cells were treated with 20 µM isoproterenol (ISO) with (BB+ISO) or without 10µM berberine (BB). D) Cultured H9c2 cells were treated with 20 µM phenylephrine (PE) with (BB+PE) or without 10µM berberine (BB). Values are expressed as Means ± S.E.M are shown. *, **, and *** indicate significant difference at $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively in average values as compared to control. # indicates significant difference at $p < 0.05$ between agonist-treated group in the presence of berberine.

Effect of berberine and hypertrophic agonists on proliferation
of H9c2 cells

As shown in figure 4.5, exposure to 2 µM AVP with or without 1 µM SR 49059
and 10 µM berberine did not alter the number of H9c2 cells. Additionally, treatment
of cultured H9c2 with insulin-growth factor II, (IGF-II, 20 nM), phenylephrine (PE,
20 µM), and isoproterenol (ISO, 20 µM) with or without 10 µM berberine did not
show any significant effect on number of cells relative to control. Even though H9c2
cells divide in culture, the rate of proliferation was unaffected by any of the various
agents. Therefore, the changes in protein content with various agents can not be
explained by changes in numbers of cells, and is consistent with alteration in cellular
hypertrophy.
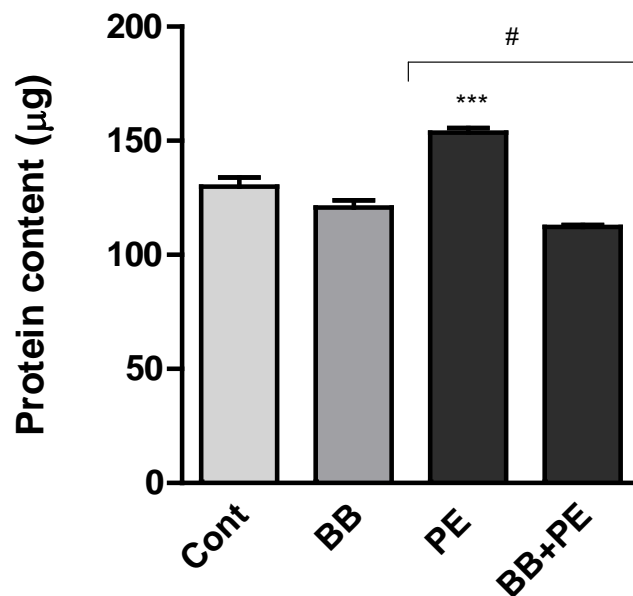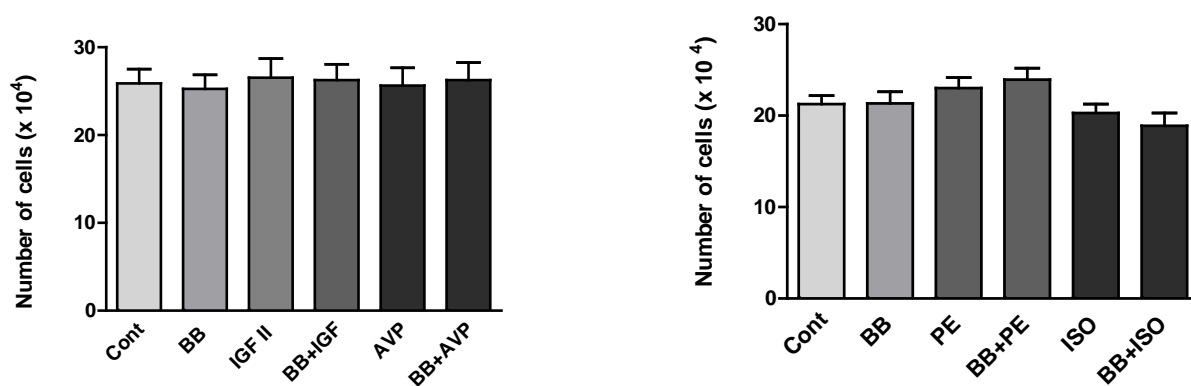
Figure 4.5 *Effect of hypertrophic agonists on number of H9c2 cells pretreated with berberine.* Cultured H9c2 cells were treated with 2 µM arginine vasopressin (AVP), 20 nM insulin-growth factoe (IGF II), 20 µM phenylephrine (PE) and 20 µM isoproterenol (ISO) with or without 10 µM berberine (BB). Values are expressed as Means ± S.E.M are shown.

## Discussion

Berberine, a basic chemical component of coptis, has been used orally for various parasitic and fungal infections and as or antidiarrheal. Based on some Chinese research data, berberine and coptis as well as its extracts are reported to have widespread effects on the cardiovascular system as antihypertensives, anti-arrhythmic, reduction of heartbeat and increased contractile force of failed heart (Shen, 1997). In a previous study, it was shown that berberine could improve the damaged heart function of chronic heart failure rats caused by aortic banding and was effective in inhibiting the development of cardiac hypertrophy (Hong et al., 2002). In another study, it is indicated that the inhibitory effect of berberine on cardiac hypertrophy is associated with modulation of sympathetic nerve activity. Berberine decreased plasma noradrenaline level and the adrenaline level both in plasma and left ventricular tissue of experimental cardiac hypertrophy rats (Hong et al., 2003). Therefore, in animal studies berberine was effective in blocking myocardial hypertrophy which is consistent with our data in H9c2 cells.

In this study, we demonstrated that berberine has a potent inhibitory effect against hypertrophy of H9c2 cells. Hypertrophic agonists such as insulin-growth factor II, phenylephrine, isoproterenol, and arginine vasopressin significantly increased cell size and protein content of H9c2 cells, which is consistent with previous studies (Huang et al., 2004; and Hwang et al, 2006). Berberine significantly inhibited the increase in cell size induced by all types of hypertrophic agonists tested in the present study in a concentration-dependent manner. This result was confirmed again by

showing that berberine prevented the hypertrophic agonists-induced increase in protein content to the control level. These results suggest that berberine has potent anti-hypertrophic effects in H9c2 cells. However the mechanism(s) of the anti-hypertrophic effect of berberine is unclear.

A variety of factors, such as angiotensin II, norepinephrine, endothelin-1 (ET-1), insulin-like growth factor (IGF)-I, interlerkin-1$\beta$, and phenylephrine, are capable of inducing cardiomyocyte hypertrophy (Sugden and Clerk., 1998). These extracellular factors act at transmembrane receptors to trigger a series of intracellular signalling events which ultimately promote hypertrophy. For example, $\alpha_1$-adrenergic agonists are identified to be prime hypertrophic agents (Guo et al., 1998; Sugden and Clerk., 1998). There are different events that characterize the growth response of cardiomyocytes to $\alpha_1$–adrenoceptor stimulation. $\alpha_1$–adrenoceptors activate protein synthesis and also induce changes in the pattern of gene expression such as ANP (Schluter and Piper., 1999).

Berberine and its derivatives showed the potential to antagonise $\alpha$-adrenergic receptors (Shen, 1997; Olmez et al., 1992). For example, in rat isolated mesenteric arteries, berberine was a non-competitive antagonist against phenylephrine-induced contraction (Ko et al, 2000). It was also suggested that a protoberberine alkaloid is a competitive antagonist of vasoconstrictory $\alpha$1-adrenceptors in peripheral small arteries (Lei et al, 1999). In rat anococcygeus muscle, the response to phenylephrine was competitively blocked by berberine, suggesting $\alpha$-antagonistic activity of this compound (Olmez and Ilhan., 1992). In the rabbit aorta, berberine blocked the effect of phenylephrine, indicating that berberine is a competitive antagonist of vascular

postsynaptic $\alpha_1$-adrenoceptors (Olmez et al., 1992). Therefoe, there is a possibility that berberine may antagonize phenylephrine-induced hypertrophy via $\alpha_1$- receptors.

Many studies reveal that an increase in intracellular $Ca^{2+}$ is a primary signal for cardiac hypertrophy. Various hypertrophic agonists including angiotensin II, endothelin-1, phenylephrine, and myocyte stretch increase $Ca^{2+}$ and the release of $Ca^{2+}$ from internal stores (Olson et al., 1999; Brostrom et al., 2000). Berberine reportedly induced endothelium-independent vascular relaxation via blocking the release of $Ca^{2+}$ from internal stores (Chiou et al., 1998). The neutral sulfate of berberine prevented abnormal calcium cycling in cardiomyocytes (Yang et al., 2006). Thus, reducing intracellular $Ca^{2+}$ may explain the anti-hypertropic effect of berberine against multiple agents.

A previous report demonstrated that berberine could have inhibitory effect on smooth muscle cell regrowth in an in vitro injury model. The suppressive effect may be explained by the observed decrease in the expression levels of mitogen-activated protein kinase 1/2 (MEK1/2), and extracellular signal-regulated kinase (ERK1/2)-dependent transcription factors Egr-1 and c-Fas, which are related to growth factor activation (Liang et al., 2006).

In another system, Lee et al (2006) have shown that berberine could inhibit vascular smooth muscle cell growth after angiotensin II or epidermal growth factor stimulation, again suggesting a common mechanism downstream of receptors coupled to increased intracellular $Ca^{2+}$ levels. The inhibitory effect of berberine on vascular smooth muscle cell regrowth was suggested to occur via decreasing Akt activation and phosphorylation (Liang et al., 2006), another possible point of convergence.The

AKT pathway is shared by different hypertrophic agents. Therefore, the effectiveness of berberine in inhibiting the response to these stimuli might indicate inhibition of their common intracellular pathway potentially in combination with antagonism at certain receptor sites.

In conclusion, our findings have provided the first scientific evidence that berberine, a pure compound from traditional Chinese herbal medicine, Huanglian, could have an inhibitory effect on hypertrophy of a cardiac cell model, H9C2 cells. Our observations suggest that berberine, or synthetic derivatives with greater bioavailability, may be potentially useful in therapeutic efforts to control hypertrophic cardiomyopathies. However, the results reported here should be assessed with further animal studies.

Chapter 5:  General conclusion

In the United States there has been an increase in the number of people who are using herbal medicines to over 10% per year. This large increase in the use of these products makes it crucial for physicians to be aware of some of the commonly used herbs. Physicians often express concerns about use of herbal therapies. Many have little or no training in herbal therapy, are not sure of their effects, adverse reactions, or interactions with other medications. Among natural products, hawthorn was selected to evaluate its pharmacological effects. Therefore, clinical studies, in vivo animal studies and in vitro studies were selected to answer some questions such as; what effects of hawthorn have been demonstrated in clinical studies? What mechanisms account for the effects of hawthorn on the cardiovascular system? What components of hawthorn are important to its action? (Zapatero, 1999).

Hawthorn has been approved and registered as a therapeutic agent for the treatment of coronary heart disease and congestive heart failure in some countries in Europe such as Germany. Hawthorn extract is as an alternative therapy for different cardiovascular diseases including hypertension, hyperlipidemia, angina and congestive heart failure. Hawthorn's popularity in the United States is documented at least back to1896 (Chang et al., 2005; Veveris et al., 2004). Recently, hawthorn is regaining attention for its potential cardiac stimulant and protective properties. One of the reasons for this attention is the narrow therapeutic index of cardiac glycosides; accidental poisoning is common and medical use is considered to be dangerous even though common in CHF therapy. Therefore, it is important that research on the

cardiovascular effects and safety of hawthorn and its active compounds, as well as different parts of hawthorn plant, be continued, especially clinical investigations.

The present results demonstrated that crude hawthorn leaf (HL) extract has significant negative chronotropic activity towards atrial cardiomyocytes, but significantly increases the contraction rate of ventricular cells. The direct negative chronotropic effects of hawthorn tablet (HT) and fraction 3 (F3) extracts, similar to the positive control carbachol (CCh), were greater in atrial cardiomyocytes than in the ventricular cells. Muscarinic receptor antagonists, atropine and himbacine, blocked the negative chronotropic effects of hawthorn extracts; competition binding studies suggest that F3 extract contains a potential ligand(s) for muscarinic receptors in mouse heart membranes. Therefore, these data support our hypothesis that hawthorn preparations produce a decrease in contraction rate of cardiomyocytes via muscarinic receptor activation. To our knowledge, this is the first report to show that a muscarinic agonist-like activity may be present in hawthorn extracts.

Chemically, hawthorn preparations have many different chemical constituents, including flavonoids (rutin, hyperoside, vitexin rhamnoside), procyanidins, other polyphenols, crataegolic acid, ursolic and oleanolic acids (Chang et al., 2005 ;Fong and Bouman, 2002 ;Veveris et al., 2004 ;Celebi Kocyildiz et al., 2006 ;Tankanow et al., 2003; Quetttier-Deleu et al., 2003; Ju, 2005). Additional minor constituents are still being identified in hawthorn preparations. After fraction 3 was utilized for a variety of activity assays, insufficient material remained for a thorough investigation of the responsible cardioactive component. Therefore, a larger scale extraction and further purification steps will be necessary to isolate, purify and identify the

compound(s) which contribute to the negative chronotropic effect of hawthorn extracts in neonatal murine cardiomyocytes and which bind to muscarinic receptors.

It is also apparent that different preparations of hawthorn are appearing more and more in accepted treatment regimens, and therefore a more complete understanding of their actions on a cellular level is warranted. Conflicting reports are appearing with regard to hawthorn and heart failure, with some papers stating that hawthorn extract is clinically effective in CHF patients, while in another study it did not show any beneficial effects when given with standard drug therapy.

Besides hawthorn, berberine is found to have widespread effects on the cardiovascular system as antihypertensives, antiarrhythmics, increasing contractile force of failing heart, and as negative chronotrophic agents (Shen, 1997). However, the mechanism(s) responsible for berberine's negative chronotropic activity is unclear.

The present study showed that: (a) in neonatal murine cardiomyocytes, berberine had a significant negative chronotropic effect, (b) berberine did not possess β-adrenoceptor blocking action, (c) atropine, himbacine, or AF-DX 116, muscarinic receptor antagonists, blocked the direct negative chronotropic activity of berberine, (d) PTX pretreatment significantly reduced the negative chronotropic effect of berberine;  and (e) berberine bound to muscarinic receptors in mouse heart and M2 receptor-transfected HEK cell membranes with micromolar affinity, similarly to the classic agonist, carbachol. In summary, these data support our hypothesis that berberine decreased the contraction rate of neonatal mouse cardiomyocytes via muscarinic receptor activation. To our knowledge, this is the first report to show that

berberine has efficacy at M2 muscarinic receptors. These data are consistent with a previous study in porcine brain in which berberine clearly binds to muscarinic receptors with an $IC_{50}$ of 1 μM (Schmeller et al., 1997). Our results suggest that activation of muscarinic receptors is the major factor responsible for the negative chronotrophic effect of berberine in cultured cardiomyocytes.

Additionally, we demonstrated that berberine has a potent inhibitory effect against hypertrophy of H9c2 cells. In the present study, hypertrophic agonists such as insulin-growth factor II, phenylephrine, isoproterenol, and arginine vasopressin significantly increased cell size and protein content of H9c2 cells, which is consistent with previous studies (Huang et al., 2004; Hwang, 2006). Berberine significantly inhibited the increase in cell size induced by almost all types of hypertrophic agonists tested in the present study in a concentration-dependent manner. This result was confirmed again by showing that berberine prevented the hypertrophic agonists-induced increase in protein content to the control level. These results suggest that berberine has potent anti-hypertrophic effects in H9c2 cells that must be downstream of various receptors and converging on a common mechanism. However, the mechanism(s) involved in the anti-hypertrophic effect of berberine remains to be determined.

In conclusion, our findings have provided the first scientific evidence that berberine, a pure compound from traditional Chinese herbal medicine, Huanglian, could have an inhibitory effect on hypertrophy of H9C2 cells. Our observations suggest that berberine or a derivative may be potentially useful in therapeutic efforts

to control hypertrophic cardiomyopathies; however, the results reported here should be assessed with further animal studies.

Several studies suggest that herbal medicines can serve as an alternative or a complementary medicine for treatment of cardiovascular diseases. The research for active principles from herbal medicines should enable us to develop new drugs for treatment of cardiovascular diseases. Although the pharmacological activities of some of the active principles are known and some of them unknown, their mechanisms of action need to be elucidated in detail. On the other hand, researchers hold different views on whether the herbal medicines can exert efficient beneficial effects due to their slow pharmacologoical activities or poor bioavailabilities. Yet it is important to understand the mechanism of the complex actions of herbal medicines that are used for treatment of cardiovascular diseases. Like any other drugs, treatment with herbal medicine will be made more effective if specific formulations can be developed (Ho and Jie, 2007 Rodriguez et al., 2008).

Active components of herbal medicines clearly show that they can be used in treatment of diseases such as cardiovascular diseases. Their mode of action is generally associated with antioxidant activity through its scavenging capability of free radicals. Specific signal transduction pathways that are associated with reduction of oxidative stress are believed to be drug targets of for the treatment of cardiovascular diseases with either herbal formulations or individual principles. The study of the biologic activity of active principles from herbal products would enable us to get a better understanding of their therapeutic benefits and its mechanism of action (Ho and

Jie, 2007), even though their adverse effects or the interactions with other

medications are not negligible.

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