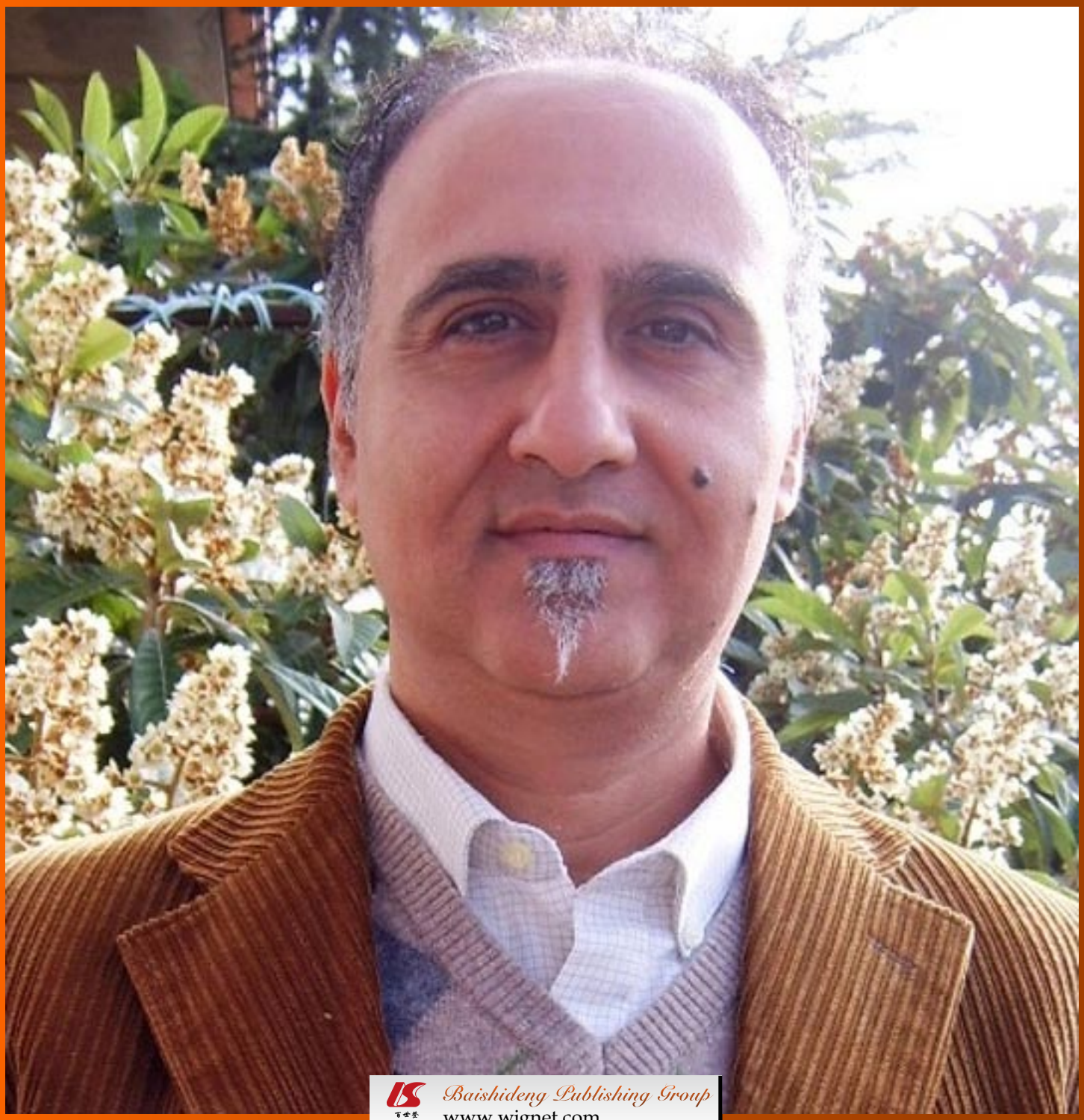
ISSN 2220-6132 (online)

World Journal of *Translational Medicine*

World J Transl Med 2013 December 12; 2(3): 32-82



Baishideng Publishing Group
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WJTM World Journal of Translational Medicine

A peer-reviewed, online, open-access journal of translational medicine

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WJTM
World Journal of Translational Medicine

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Four-monthly Volume 2 Number 3 December 12, 2013

World Journal of Translational Medicine
Volume 2 Number 3 December 12, 2013

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NAME OF JOURNAL
World Journal of Translational Medicine

ISSN
ISSN 2220-6132 (online)

LAUNCH DATE
June 12, 2012

FREQUENCY
Four-monthly

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PUBLISHER
Baishideng Publishing Group Co., Limited
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315-321 Lockhart Road, Wan Chai,
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Fax: +852-6555-7188
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http://www.wjgnet.com

PUBLICATION DATE
December 12, 2013

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/2220-6132/g_info_20100722180909.htm.

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World Journal of Translational Medicine

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bpgoffice@wjgnet.com
doi:10.5528/wjtm.v2.i3.32

World J Transl Med 2013 December 12; 2(3): 32-35
ISSN 2220-6132 (online)

FIELD OF VISION

Peptide-based boronates: How to achieve tissue specificity in anticancer therapy

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Received: July 24, 2013 Revised: September 4, 2013
Accepted: September 13, 2013
Published online: December 12, 2013

Abstract

Dipeptidyl boronic acids are suitable candidates for the design of "pro-soft" drugs because recent studies have proven that these acids undergo a pH-dependent cyclization equilibrium, generating an inactive cyclic form under physiological conditions. Dipeptidyl boronic acids possess a wide range of potential targets, and the 26S proteasome appears to be one of the main targets. This multicatalytic complex is involved in intracellular protein turnover and is overexpressed in certain pathological conditions, such as malignancies, autoimmune diseases and neurodegenerative diseases. Bortezomib is the first-in-class derivative approved by the Food and Drug Administration for the treatment of hematological malignancies (*i.e.*, relapsed and refractory multiple myeloma and mantle cell lymphoma) but is inactive against solid tumors due to an insufficient tissue distribution. The present study suggests a possible strategy for enhancing the *in vivo* performance of dipeptidyl boronic acids endowed with promising proteasome-inhibiting properties and their applicability as anticancer agents. In particular, dipeptidyl boronic acids might have a fruitful application as pro-soft drugs when an appropriate recognition motif serves as a substrate for a tumor-specific protease, generating the active form of the drug in situ and preventing systemic side effects after diffusion through cells and tissues.

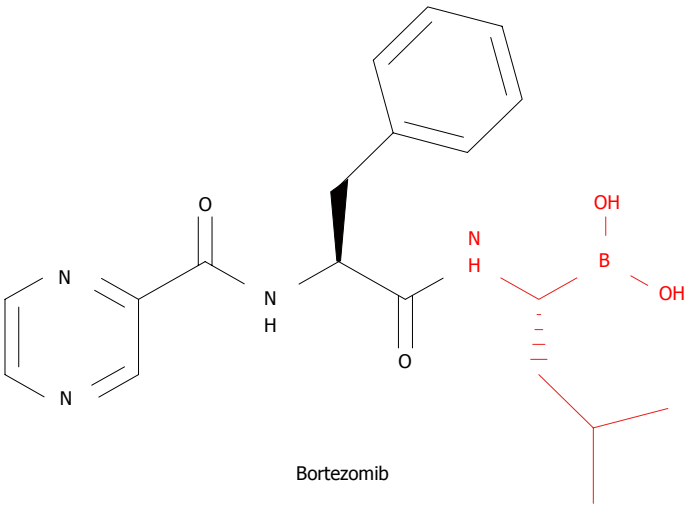
Key words: Peptide boronates; Proteasome inhibitors; Anticancer therapy; Pro-soft drug; Solid tumors

Core tip: The design of "pro-soft" drugs is a promising strategy for enhancing the tissue specificity of drugs and for avoiding systemic adverse effects. This strategy might be applied to dipeptidyl boronic acids for use as proteasome inhibitors.

Micale N. Peptide-based boronates: How to achieve tissue specificity in anticancer therapy. *World J Transl Med* 2013; 2(3): 32-35 Available from: URL: http://www.wjgnet.com/2220-6132/full/v2/i3/32.htm DOI: http://dx.doi.org/10.5528/wjtm.v2.i3.32

COMMENTARY ON HOT TOPICS

The approval of the 20S proteasome inhibitor bortezomib (Velcade®) by the Food and Drug Administration in 2003 for the treatment of multiple myeloma marked a historical moment in proteasome research[1]. This relatively small drug is a dipeptide boronate protected at its *N*-terminus by a pyrazinyl group that preferentially inhibits the chymotrypsin-like (β5) enzymatic sites of the proteasome, which are mainly involved in intracellular protein breakdown (Figure 1)[2]. Later, in 2006, the drug was approved for the treatment of another hematological malignancy, mantle cell lymphoma, further underscoring the importance of the 20S proteasome as a drug target in anticancer therapy[3]. Since this time, several research groups have focused their efforts on attempting to obtain similar compounds with a better pharmacological profile compared with this first-in-class derivative[4-11]. In fact, bortezomib presents certain shortcomings as a therapeutic agent, including its route of administration (intravenous bolus); its limited activity against solid tumors; the development of tumor resistance to bortezomib; and the drug's dose-limiting adverse effect (reversible peripheral



Bortezomib

Figure 1 Structure of the proteasome inhibitor bortezomib. The amino acid residue marked in red at the C-terminus (boroLeu unit) is responsible for its β5-preferring (chymotrypsin-like) activity and represents the electrophilic moiety that covalently traps the catalytic Thr1Oγ with a slow off-rate.

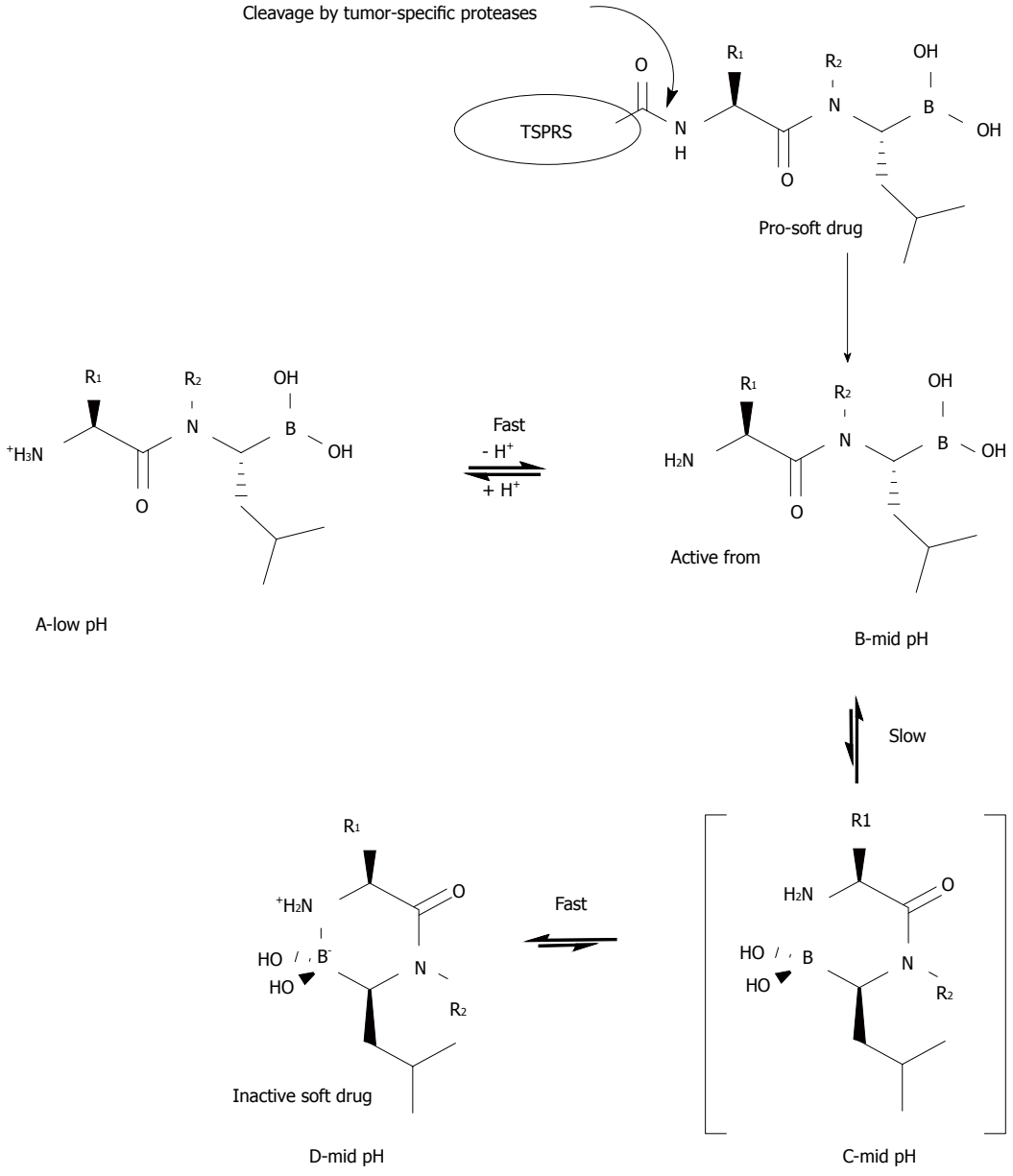


Figure 2 Activation and pH-dependent cyclization of dipeptidyl boroLeu. A tumor-specific protease recognition sequence (TSPRS) is attached to the *N*-terminus of the dipeptidyl boroLeu. The dipeptidyl boroLeu is released in its active form by a tumor-specific protease and undergoes a pH-dependent cyclization equilibrium. In the present study, low pH = 2 and mid pH = 7.6.

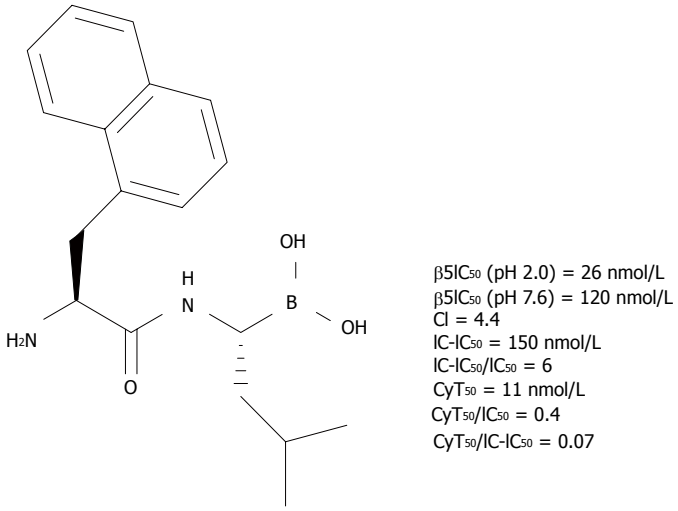


Figure 3 Structure and activity of the most interesting dipeptidyl boroLeu developed by Milo *et al*[14]. IC-IC_{50}/IC_{50} ratio → a measure of cell permeability, for which a value of 1.0 corresponds to 100%; CyT_{50}/IC_{50} ratio → the relationship between cytotoxicity and inhibition, for which a value > 1.0 indicates that more than 50% inhibition is needed to kill 50% of the cells. CI: Cyclization index; IC: Intracellular; CyT_{50}: Cytotoxicity.

neuropathy), caused primarily by "on-target" inhibition of the proteasome in normal cells[12].

Based on the current literature, a dipeptide sequence represents the smallest chemical frame that affords efficacious proteasome inhibition, and a C-terminal Leu-boronic acid moiety ensures specificity for the β5 catalytic sites of the proteasome and influences the nature of the inhibition (covalent and slowly reversible; Figure 2). Moreover, the poor affinity between boron and sulfur atoms makes peptide boronates targetable by human cysteine proteases and suitable for application *in vivo*[13].

The inefficacy of bortezomib against solid tumors prompted Milo *et al*[14] to evaluate a strategy for enhancing the tissue specificity of various dipeptidyl Leu-boronic acids and to verify the applicability of this strategy. The strategy consists of designing a longer peptide-based prodrug in which the active boroLeu dipeptide fragment at the C-terminus can be released by a tumor-specific protease. This tumor specificity is relative because these proteases (generally glycoproteins) are also present in normal cells/tissues at a lower level. Regarding solid tumors, several specific proteases may undertake this activation role (*e.g.*, fibroblast activation protein, prostate-specific antigen, and prostate-specific membrane antigen)[15-17]. However, the main issue with this strategy is represented by the free *N*-terminal amino group that is generated after the peptidic cleavage. Are the resulting dipeptides of boroLeu sufficiently potent, cell penetrating, and stable against degradation by cellular peptidases? Based on previous studies, the same authors knew that free NH_2-terminal dipeptidyl boroPro undergoes a pH-dependent and reversible cyclization reaction that implies the nucleophilic attack of the amino group on the boron atom[18]. Milo *et al*[14] demonstrated that this pH-dependent equilibrium also exists for the dipeptides of boroLeu, although the cyclization is relatively modest compared with that exhibited by the dipeptides of boroPro. At physiological pH, the inactive cyclic-form D predominates, whereas at low pH, the active open-form A prevails. The loss of pharmacological activity with time is characteristic of compounds termed "soft drugs", and the above-mentioned pH-dependent equilibrium might be exploited to obtain "pro-soft" drugs. The pro-soft drug will act as a substrate for a tumor-specific protease, which in turn will release the drug in its active open form; cyclization subsequent to release might limit adverse effects as excess inhibitor diffuses from the tumor site. Notably, tumor cellular pH is more acidic than the pH in a normal cell, which might favor the pH-dependent equilibrium of "pro-soft" drugs to selectively gain activity in tumor cells. The strategy of generating the active form of a dipeptidyl boroLeu from a pro-soft drug and its pH-dependent cyclization reaction are depicted in Figure 2.

Given this information, Milo *et al*[14] synthesized and evaluated a wide series of dipeptides of boroLeu *in vitro*, demonstrating that despite the presence of a free amino group, these small molecules were comparable with bortezomib in terms of potency and cell-penetrating ability. Furthermore, these molecules were sufficiently cytotoxic and stable against degradation by aminopeptidases. The substitution pattern at P_2 consisted of both natural and non-natural amino acids[14]. Structural and other significant data for the most druggable candidate (P_2 = 1-naphthylalanyl) in the construction of pro-soft drugs are reported in Figure 3.

Pro-soft drug design is a strategy that, in principle, can be applied to wide variety of other inhibitors and targets. However, this strategy deserves special attention in cases, such as the case presented here, in which the tissue specificity of the first target (*i.e.*, the protease that causes the removal of the recognition sequence and the release of the active drug) might play a decisive role in reducing side effects arising from the systemic activity of the drug. The pro-soft strategy is also more reliable when the activating and target enzymes are not the same, allowing enough time for the formation of the soft drug and its correct measurement. It is well known that dipeptidyl boronic acids may act as substrates for a wide range of enzymes[14,18], so the potential for the application of these molecules in drug design is very high. Proteasome inhibition may represent the research area with the best potential, and this strategy, when applied to dipeptidyl boronic

acids, may extend the application of the proteasome inhibitors currently under study to solid tumors.

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P- Reviewers: Dou QP, Morais C **S- Editor**: Song XX
L- Editor: A **E- Editor**: Wu HL

World Journal of Translational Medicine

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.5528/wjtm.v2.i3.36

World J Transl Med 2013 December 12; 2(3): 36-48
ISSN 2220-6132 (online)

REVIEW

Hypo-activity induced skeletal muscle atrophy and potential nutritional interventions: A review

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Received: May 24, 2013 Revised: September 7, 2013
Accepted: November 1, 2013
Published online: December 12, 2013

Abstract

Periods of hypo-activity result in profound changes in skeletal muscle morphology and strength. This review primarily addresses the differential impact of de-training, bed-rest, limb immobilisation and unilateral lower limb suspension on muscle morphology, strength and fatigability. The degree of muscle atrophy differs depending on the hypo-activity model and the muscles in question, with the leg and postural muscles being the most susceptible to atrophy. Hypo-activity also results in the dramatic loss of strength that often surpasses the loss of muscle mass, and consequently, the nervous system and contractile properties adapt to adjust for this excessive loss of strength. In addition, the degree of muscle strength loss is different depending on the hypo-activity model, with immobilisation appearing to have a greater impact on strength than unloaded models. There is a step-wise difference in the magnitude of muscle loss so that, even after accounting for differential durations of interventions immobilisation ⩾ unilateral lower limb suspension ⩾ bed-rest ⩾ de-training. Muscle fatigability varies between hypo-activity models but the results are equivocal and this may be due to task-specific adaptations. This review also addresses potential nutritional interventions for attenuating hypo-activity induced muscle atrophy and strength declines, in the absence of exercise. Essential amino acid supplementation stands as a strong candidate but other supplements are good contenders for attenuating hypo-activity induced atrophy and strength losses. Several potential nutritional supplements are highlighted that could be used to combat muscle atrophy but extensive research is needed to determine the most effective.

Key words: Immobilisation; Disuse; Muscle size; Muscle strength; Nutrition supplementation; Muscle fatigability

Core tip: This review summarises and compares the morphological, strength and fatigability changes in response to different models of hypo-activity. The hypo-activity models include de-training, bed-rest, immobilisation and unilateral lower limb suspension. There is a step-wise difference in the magnitude of muscle and somewhat strength losses so that, even after accounting for differential durations of interventions immobilisation ⩾ unilateral lower limb suspension ⩾ bed-rest ⩾ de-training. Muscle fatigability varies between hypo-activity models but the results are equivocal and this may be due to task-specific adaptations. This review also highlights several potential nutritional interventions for attenuating hypo-activity induced changes.

Bostock EL, Morse CI, Winwood K, McEwan I, Onambélé-Pearson GL. Hypo-activity induced skeletal muscle atrophy and potential nutritional interventions: A review. *World J Transl Med* 2013; 2(3): 36-48 Available from: URL: http://www.wjgnet.com/2220-6132/full/v2/i3/36.htm DOI: http://dx.doi.org/10.5528/wjtm.v2.i3.36

INTRODUCTION

Skeletal muscle is one of the most adaptable tissues in the body, and as such, it is capable of altering its structure in response to different levels of physical activity. Prolonged reductions in muscle activity and mechanical loading result in many physiological adaptations in skeletal muscle form and function[1-4]. Muscle atrophy (decrease in muscle mass) is seen during reduced activity (*e.g.*, sedentary behaviour, de-training)[5-8] or disuse models (*e.g.*, immobilisation, head-down tilt bed-rest)[1,3,9,10]. It is evident that the degree of muscle atrophy is not constant across muscle groups or hypo-activity models[2,3,11,12].

Simply reducing normal levels of activity can be classed as the first stage of disuse. Decrements in muscle mass and strength have been documented in trained humans undergoing de-training[5-8,13-15]. Bed-rest conditions result in the removal of normal weight-bearing forces acting on the bones of the lower limbs in the vertical position and a decrease in number and/or magnitude of muscle contractions, particularly in the postural musculature. During bed-rest, muscular contraction is still possible although it is limited and the muscular force required for producing movement is very much diminished once ground reaction forces are removed. A more rigid immobilisation can be achieved by casting a limb, resulting in more rapid decrements in muscle mass than does bed-rest alone. The final method of hypo-activity commonly reported in the literature is that of unilateral lower limb suspension (ULLS), a method of reducing habitual activity whilst causing lesser degree of inconvenience to the participants.

The purpose of this review is to assess the varying impact of different hypo-activity models on the skeletal muscle system. This is broken down into the effects of hypo-activity on muscle morphology, muscle strength and muscle fatigability. In order to provide some homogeneity in the results based on the variable duration of the hypo-activity, values are presented per week and where relevant the duration of the hypo-activity is provided in parenthesis. Exercise prescription is not always a practical prescription, even when it would be recommendable to individuals under-going immobilisation or bed-rest after trauma or illness, due to the presence of counter indications for exercise such as pain, immobilisation in a cast, *etc*. Thus, other interventions are required to attenuate losses in muscle mass and function. Therefore, this review will also discuss potential nutritional interventions for preventing the loss of muscle mass/function seen with hypo-activity, where increased physical activity is not combined with the nutritional treatment. Studies were found using search terms "bed-rest and atrophy" and "immobilisation and atrophy" in PubMed. However, this returned over 1400 hits. To focus our search criteria, only data on healthy humans were selected through the inclusion of the "human" and "clinical trial" filters in the PubMed search. This resulted in 86 studies, suitable for inclusion in the present review.

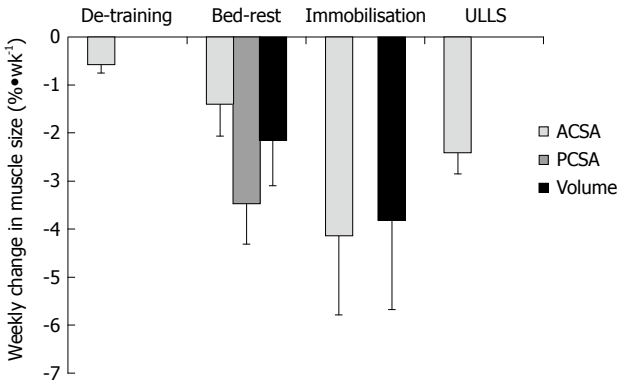

Figure 1 Relative change in muscle anatomical cross sectional area, physiological cross sectional area and volume. ULLS: Unilateral lower limb suspension; ACSA: Anatomical cross sectional area; PCSA: Physiological cross sectional area.

MUSCLE MORPHOLOGY

Muscle anatomical cross sectional area

Anatomical cross sectional area (ACSA) is the cross-sectional area of the muscle at right angles to its longitudinal axis. Muscle ACSA is a major determinant of maximum voluntary contraction (MVC) torque[16,17] and hypo-activity models have been shown to result in the decrease in this parameter. [Figure 1 shows relative change in muscle anatomical cross sectional area (ACSA), physiological cross sectional area (PCSA) and volume in response to hypo-activity models. Values are taken from the references used in the text for de-training (ACSA-40 d and 24 wk)[6,8], bed-rest (ACSA-30 d to 17 wk)[2,11,18,19] (PCSA-20 d)[1,9,20] (Volume-7 d and 32 d)[11,21], immobilisation (ACSA-9 d to 4 wk)[3,4,12,22-26] (Volume-2 wk and 4 wk)[10,12,27] and ULLS (ACSA-23 d and 4 wk)[28-31]. Where there are missing bars, this shows gaps in the literature (*i.e.*, values are not available for a parameter during a specific hypo-activity model). Values are presented as means; error bars denote SD]. Periods of detraining (24 wk) have resulted in a decrease in ACSA of the quadriceps[6]. Likewise, Narici *et al*[8] reported decreases in leg ACSA (approximately 0.7%/wk) in response to 40 d de-training.

Stricter hypo-activity models result in greater decreases in muscle ACSA. Following 30 d bed-rest Convertino *et al*[11] reported decreases in ACSA of the calf (approximately 1.1%/wk) and thigh (approximately 1.9%/wk). Similarly, a 2.4%/wk decrease in plantar flexors was found following 5 wk horizontal bed-rest[18]. Muscle group-specific adaptations have been demonstrated in skeletal muscle ACSA of the leg and lumbar musculature after 17 wk of bed-rest[2]. The plantar-flexors were more susceptible to atrophy (approximately 1.8%/wk) than the dorsiflexors (approximately 0.9% to 1.2%/wk)[2]. The intrinsic lumbar muscles atrophied approximately 0.5%/wk but there was no significant change in psoas muscle mass[2]. Rittweger *et al*[19] reported a decrease in calf muscle ACSA (approximately 2.0%/wk), which was greater than the reported decrease in the forearm ACSA (0.5%/wk) in response to 90 d bed-rest.

Immobilisation of the leg through plaster cast has shown to decrease calf ACSA (approximately 3% to 5%/wk) after just 2 wk[4,22]. Changes in quadriceps ACSA (approximately 8.3%/wk) have also been documented with as little as 10 d leg cast immobilisation[25]. Similarly, Veldhuizen *et al*[3] reported decreases in quadriceps ACSA (approximately 5.3%/wk) with 4 wk leg casting. Immobilisation of the knee using a brace has also resulted in decreases in muscle ACSA[24,26]. Fourteen days of knee-brace immobilisation has resulted in ACSA decreases of the thigh (approximately 3.1%/wk), quadriceps (approximately 2.9% to 3.8%/wk), gastrocnemius (approximately 4.7%/wk) and soleus (approximately 3.3%/wk) muscles[24,26]. Yasuda *et al*[26] found no sex-based differences in the quadriceps ACSA response to knee-brace mediated immobilisation. There is considerably less data on immobilisation-induced atrophy of the upper limb muscles. Casting of the arm for as little as 9 d has shown to decrease ACSA of the forearm (approximately 3.2%/wk)[23]. Yue *et al*[12] investigated the effect of 4 wk elbow joint immobilisation with a fibre glass cast and reported a decrease in elbow flexor ACSA (approximately 2.8%/wk).

Tesch *et al*[32] developed a model to study the effects of an unloaded limb in humans that allows for freely moveable joints but minimises load bearing. In this ULLS method, a sling suspends one lower leg and the contralateral shoe has an elevated sole to allow for a relaxed position of the unloaded limb. ULLS also results in decreases in muscle ACSA, though to a lesser degree than immobilisation. ULLS of 23 d has been reported to decrease knee extensor (approximately 3%/wk)[30] and plantar flexor (approximately 2.7%/wk)[31] ACSA. Correspondingly, Clark *et al*[28,29] reported decreases in plantar flexor (approximately 2.0% to 2.3%/wk) and knee extensor (approximately 2.0%/wk)[29] ACSA in response to 4 wk ULLS. It would therefore seem that in terms of ACSA at least, the most impactful model of hypo-activity is immobilisation.

Muscle physiological cross sectional area

PCSA is the area of the muscle at right angles to the longitudinal axis of the fibres. Muscle PCSA has been associated with the maximal force generating capacity of a muscle[33] and has been shown to decrease with bed-rest[1,9,20]. Twenty days bed-rest has been shown to decrease PCSA of the thigh (between approximately 2.7% to 3.6%/wk)[1,20]. Akima *et al*[9] described muscle group-specific adaptations, demonstrating a decrease in PCSA of knee extensor (approximately 2.5%/wk), knee flexor (approximately 4.0%/wk) and plantarflexor (approximately 4.5%/wk) muscles in response to 20 d of 6 degrees head-down-tilt bed rest. It is generally accepted that muscle losses are greater in the knee extensors than the knee flexors after unloading in humans[34]. Akima *et al*[9] demonstrated the opposite to this, which could be due to the methodology used to determine PCSA. In addition, since a muscle placed in a shortened position experiences a greater degree of atrophy than one placed in a lengthened position[35], the pattern/magnitude of disuse would therefore be expected to be modulated by both the mode of hypo-activity and the joint angle adopted in the immobilisation. Bed-rest, however, had no effect on the PCSA of the tibialis anterior[9]. The tibialis anterior experiences lower activation during habitual physical activities than other muscles such as the plantar flexor, and as such may explain the lack of decrease in tibialis anterior muscle PCSA with bed-rest. Comparisons of bed-rest to other hypo-activity models in terms of PCSA changes is not yet possible, as research is lacking with this parameter being measured.

Muscle volume

Muscle volume is a major determinant of joint torque[36] and has been shown to decrease in response to bed-rest and immobilisation models[10-12,21,27]. Muscle volume of the thigh decreases (approximately 3%/wk) with as little as 7 d bed rest[21]. Following 30 d bed rest, Convertino *et al*[11] reported decreases in calculated leg volumes of the calf (approximately 2.3%/wk) and thigh (approximately 1.1%/wk). Yue *et al*[12] investigated the effect of 4 wk elbow joint immobilisation with a fibre glass cast and reported a decrease in elbow flexor volume (approximately 2.9%/wk). A case study of a orthopaedic patient who fractured the fifth metatarsal of the right foot displayed substantial and rapid losses in muscle volume, both proximally and distally to the immobilisation site after 4 wk subsequent immobilisation[10]. The degree of muscle volume decrease varied between the different muscle sites of the triceps surae (approximately 5.5%/wk), quadriceps (approximately 6.0%/wk) and hamstrings (approximately 1.6%/wk)[10]. This is in agreement with the general acceptance that muscle volume is lost to a greater extent in the knee extensors compared to the knee flexors[34]. An age-related susceptibility to immobilization i also evident whereby, Urso *et al*[27] demonstrated different responses to 2 wk adductor pollicis (AP) immobilisation between younger and older males. AP volume decreased approximately 2.1%/wk (not significant) in young males and significantly decreased by approximately 4.8%/wk in older males[27].

Upper vs lower limb

Immobilisation through casting appears to have a greater effect on the lower limb musculature than the upper body. This is not surprising since the habitual loading of the lower extremities, because of body weight in normal ambulation and even in the absence of intended physical exertion, is far more substantial than that in the upper extremities. Understandably, this thereby affects the required threshold of decrease in muscle activity necessary to negatively impact on muscle metabolism. [Relative change in muscle ACSA, PCSA and volume in response to hypo-activity models. Values are separated into the effect of each hypo-activity model on the upper limb (UL) *vs* the lower limb (LL). The values are taken from the refer-

Table 1 Relative change in upper and lower limb muscle anatomical cross sectional area, physiological cross sectional area and volume

	ACSA_UL (%)	ACSA_LL (%)	PCSA_UL (%)	PCSA_LL (%)	Volume_UL(%)	Volume_LL (%)
De-training	-	-0.6	-	-	-	-
Bed-rest	-0.5	-1.5	-	-3.5	-	-2.1
Immobilisation	-3	-4.4	-	-	-3.3	-4.4
ULLS	-	-2.4	-	-	-	-
Mean (SD) of 4 models	-1.8 (1.8)	-2.2 (1.6)		-3.5 (0.01)	-3.3 (0.01)	-3.3 (1.6)

ACSA: Anatomical cross sectional area; PCSA: Physiological cross sectional area; ULLS: Unilateral lower limb suspension; UL: Upper limb; LL: Lower limb.

ences used in the text for de-training (ACSA_LL)[6,8], bed-rest (ACSA_UL)[19] (ACSA_LL)[2,11,18,19] (PCSA_LL)[1,9,20] (Volume_LL)[11,21], immobilisation (ACSA_UL)[12,23] (ACSA_LL)[3,4,22,24-26] (Volume_UL)[12,27] (Volume_LL)[32] and ULLS (ACSA_LL)[28-31]. Where there are missing values, this shows gaps in the literature *(i.e.*, values are not available for a parameter during a specific hypo-activity model) (Table 1)]. Forearm muscle ACSA decreased 4.1% with 9 d arm casting[23], whereas, a similar period of immobilisation of the lower limb with 10 d casting resulted in an 11.8% decrease in quadriceps ACSA[25]. Similarly, with longer periods of immobilisation the effect seems to be greater in the lower limbs. In response to 4 wk elbow joint casting, Yue *et al*[12] reported an 11.2% decrease in elbow flexor ACSA, whereas, Veldhuizen *et al*[3] reported a 21% decrease in quadriceps ACSA in response to 4 wk leg casting.

Intramuscular adipose tissue

Using signal intensity analysis of lower limb magnetic resonance images (MRI). Manini *et al*[37] discriminated between the relative changes in adipose and skeletal muscle tissue resulting from a 4 wk period of ULLS. In addition to the characteristic reduction in muscle ACSA, there was a concomitant 15% increase in intermuscular adipose content after 4 wk of lower limb suspension[37]. Thus, these findings suggest, that hypo-activity induced alterations in skeletal muscle morphology goes beyond muscle atrophy alone.

Summary

Together, these findings show that the extent of muscle atrophy differs depending on the hypo-activity model. Certain factors may modulate the differential responses to hypo-activity models (*e.g.*, age, nutritional status). Indeed, both Kortebein *et al*[38] and Urso *et al*[27] suggested that older individuals experience greater losses in muscle mass when compared to younger individuals. A change in nutritional status, whether it is due to physiological changes directly caused by hypo-activity or to altered behaviour that is caused by hypo-activity and leads to changes in diet, could affect the physiological systems in question. The above also suggest that the degree of muscle atrophy differs between muscle groups, with the leg and postural muscles being most susceptible to atrophy. This is likely to be due to the comparatively substantial decrease in habitual weight-bearing forces applied to the lower limb during hypo-activity. Hypo-activity not only decreases muscle content, but also impacts on the intrinsic composition of the said skeletal muscle through increased adiposity[37] and altered muscle architecture[39].

The decrease in muscle mass seen with hypo-activity may be the result of an imbalance between protein synthesis and protein breakdown[40-42]. In response to 14 d simulated microgravity, Ferrando *et al*[40] reported a loss of lean muscle mass, accompanied with a 14% decrease in protein synthesis and no change in protein breakdown. Similarly, Gibson *et al*[41] reported a marked fall in muscle protein synthesis in response to 7 wk leg immobilisation. A shorter period of immobilisation (21 d) provided little evidence of increases in mRNA for catabolic enzymes or increases in enzyme activity during this period[43]. However, there is some evidence to suggest that increases in catabolic potential do occur, and that this event happens very quickly (48 h) after immobilisation[42]. Nevertheless, collectively the evidence suggests that protein breakdown is unlikely to be a key modulator in the process of muscle atrophy occurring during immobilisation in humans[44,45].

The molecular signalling responses to de-training are only just beginning to be investigated, and to date, only changes in metabolic proteins have been reported in human skeletal muscle[46,47]. With bed-rest, Ogawa *et al*[48] reported increased mRNA expression of the E3 ligases, Cbl-b and Atrogin-1 in response to 20 d bed-rest. This was accompanied by a threefold increase in ubiquitinated proteins[48]. Investigation into the effects of limb immobilisation on cell signalling in humans is limited. Modest changes in mRNA for many genes in the first 2 d after immobilisation have been reported but these changes do not affect protein levels of most transcripts[42]. However, the Akt protein synthesis pathway and extracellular matrix components seem to be affected within 48 hours of immobilisation[42]. Chen *et al*[49] and Jones *et al*[50] reported increases in the E3 ligases, Atrogin-1 and MuRF-1 in response to 11 to 14 d immobilisation in humans. These changes were not seen with 48 h immobilisation[42] and are therefore thought to only occur after long duration (days rather than hours) immobilisation. Increased metallothionein expression in human skeletal muscle fibres has been associated with exposure to physiological stress, which results in elevated levels of reactive oxygen species

(ROS)[51]. Urso *et al*[42] reported a more than two-fold increase in metallothioneins in human skeletal muscle with 48 h of immobilisation. However, neither Chen *et al*[49] nor Jones *et al*[50] identified changes with longer periods of immobilisation. This may suggest that metallothioneins are increased in the first few days of hypo-activity to prevent ROS-mediated DNA or cellular damage. de Boer *et al*[43] investigated the effects of ULLS on gene expression and cell signalling. They reported increased expression of mRNA for MuRF-1 by approximately 3 fold after 10 d without changes in MAFbx or tripeptidyl peptidase Ⅱ mRNA, but all decreased between 10 and 21 d[43]. These authors concluded that both myofibrillar and tendon protein synthetic rates show progressive decreases during 21 d of disuse; in muscle this is accompanied by decreased phosphorylation of FAK, with no marked increases in genes for proteolytic enzymes[43]. Overall, whilst it is clear that cell signalling responses differ between hypo-activity models; further research is needed to provide a definitive description of the timing, magnitude and nature of these molecular adaptations.

MUSCLE STRENGTH

The associated decline in strength through hypo-activity can be best described based on the mode of assessment. Both isometric and dynamic strength have been reported to decline with hypo-activity, the relative magnitude of which appears to largely reflect the patterns of atrophy described above.

Isometric strength

Hypo-activity models alter muscular isometric torque. After 40 d de-training, Narici *et al*[8] reported a decrease in knee extension isometric MVC (approximately 2.1%/wk). Similarly, maximum isometric quadriceps strength has been reported to decrease with 90 d de-training (approximately 1.3%/wk)[5]. More dramatic losses in isometric torque are seen with stricter hypo-activity models. Bed-rest models have been shown to decrease maximum voluntary force of plantar flexion (approximately 7.5%/wk)[52] and knee extensor torque (approximately 4.1% to 5.0%/wk)[53]. Correspondingly, Kawakami *et al*[1] showed a decrease in muscle force for knee extension (approximately 3.8%/wk) with 20 d bed-rest.

Studies using 2 wk of cast immobilisation have reported decreases in triceps surae isometric MVC torque (approximately 8.5 and 12%/wk)[4,54]. A discrepancy between the two studies may be due to the degree of immobilisation. Gondin *et al*[54] simply immobilised the ankle joint, whilst, White *et al*[4] utilised a full leg cast. Knee-brace mediated immobilisation has resulted in a decrease in knee extensor and plantar flexion isometric strength (approximately 11.2 and 12.7%/wk, respectively)[24]. Knee-cast mediated immobilisation resulted in a slightly larger decrease in isometric leg strength (approximately 15.7%/wk)[55]. Christensen *et al*[22] utilised a knee-to-toe plaster cast and reported a decrease in isometric calf muscle strength (approximately 4.5%/wk). Studies using casting to immobilise the elbow joint have found decreases in isometric MVC of the elbow flexors (approximately 5.3% to 8.8%/wk)[12,56,57], and a decrease in the maximum load that could be lifted[12]. A more dramatic decrease in isometric MVC torque has been reported in the flexors and extensors of the wrist (approximately 22.8% to 25.3%/wk) in response to immobilisation[23,58].

With ULLS, isometric torque appears to be affected to a lesser degree than with immobilisation models. An explanation for the above observation may be that ULLS removes weight-bearing but allows for freely moveable joints (hence a degree of muscular activity) whereas immobilisation is a more rigid model that does not allow joint movement (hence a greater restriction of muscular activity). Studies have reported plantar flexor isometric MVC torque to decrease (approximately 5% to 7 %/wk) with ULLS[28,31]. With ULLS, increased fluctuations in plantar flexion (approximately 3%/wk) and knee extension (approximately 5.5%/wk) isometric force have been demonstrated[29].

Isokinetic strength

In addition to the established decline in isometric strength (torque and force), hypo-activity models (de-training, bed-rest, immobilisation and ULLS) also result in reductions in dynamic torque outputs. Hypo-activity models also result in changes to dynamic torque outputs. After 14 d de-training isokinetic eccentric and concentric knee extension force has been shown to decrease by approximately 6% and 1.2%/wk, respectively[7]. With as little as 14 d bed-rest decrements in knee extensor 1 repetition maximum (approximately 4.5%/wk) are seen along with a fall in MVC (approximately 7.5%/wk)[59]. After 6 wk bed-rest maximum voluntary concentric knee extensor torque was shown to decrease uniformly across angular velocities (approximately 4.1% to 5.0%/wk)[53]. Muscle-specific adaptations are evident with bed-rest, as shown by Dudley *et al*[60] who reported a decrease in concentric and eccentric isokinetic knee extensor peak torque (approximately 4.4%/wk), with no alterations in knee flexors in response to 30 d 6 degrees head-down bed-rest. Again muscle-specific adaptations were demonstrated by LeBlanc *et al*[18] who reported a decrease in plantar flexor concentric isokinetic strength (approximately 2.6%/wk) and no change in the isokinetic strength of the dorsiflexors with 5 wk bed-rest. As with the knee extensors *vs* knee flexors difference in sensitivity to hypo-activity alluded to above, the plantar flexor muscles experience a greater level of recruitment during gait than the tibialis anterior. Thus, habitual muscle recruitment prior to hypo-activity would appear to be a large determinant of the relative magnitude of hypo-activity-induced changes.

Results from lower limb immobilisation models indicate that short-term immobilisation is associated not only with atrophy but with a diminished capacity of the muscle to perform both concentric and eccentric strength[23,55]. Lower limb casting results in a dramatic

decrease in isokinetic quadriceps strength (approximately 29.1%/wk)[25]. There is evidence that the effect of leg cast immobilisation on isokinetic strength of the knee extensors and flexors is greater in the knee extensors, demonstrated by a fall in peak torque of approximately 13.3%/wk for the knee extensors and approximately 3.3%/wk for knee flexors[3]. Cast immobilisation of the arm also results in decreased concentric (approximately 6.9% to 16.9%/wk) and eccentric (approximately 9.7% to 14.4%/wk) strength for flexion, extension, pronation and supination of the wrist[23].

Less dramatic decreases in isokinetic strength are seen with ULLS compared to immobilisation. de Boer *et al*[30] found a decrease in isokinetic knee extensor torque in response to 23 d ULLS (approximately 6.4%/wk). Similarly, after 4 wk ULLS mean average peak isokinetic torque is decreased (approximately 4.3%/wk)[61]. With as little as 14 d ULLS, a decrease in peak isokinetic torque (approximately 5% to 8.6%/wk) and total work performed (approximately 7.5% to 10.0%/wk) by knee extensors and flexors was reported[62].

Strength vs size changes

There is evidence to suggest that decreases seen in strength in response to hypo-activity models are greater than the changes seen in muscle size. With de-training the loss in leg muscle ACSA (approximately 0.7%/wk) was not as great as the decrease seen in knee extension MVC (approximately 2.1%/wk)[8]. Similarly, in bed-rest Kawakami *et al*[1] suggested that the decrease in knee extension mean muscle force (approximately 3.8%/wk) seen after 20 d head down bed-rest was related more to changes in neural activation to those in PCSA (approximately 2.7%/wk). Correspondingly, Berg *et al*[53] suggested that the decline seen in strength (approximately 4.1% to 5.0%/wk) could not be entirely accounted for by decreased ACSA (approximately 2.3%/wk), and that the strength loss could also be due to factors resulting in decreased neural input to muscle and/or reduced specific tension of muscle, as evidenced by a decreased torque to EMG ratio. Discrepancies between decreases in muscle size and muscle strength have also been reported in upper and lower immobilisation studies. White *et al*[4] reported an approximately 5%/wk decrease in muscle ACSA whilst triceps surae MVC decreased approximately 12%/wk. Additionally, the upper limb decreases in forearm ACSA (approximately 3.2%/wk) were much smaller than those reported in forearm flexor and extensor strength (approximately 22.8% to 25.3 %/wk)[23]. Again, in ULLS models muscle torque (approximately 5% to 7%/wk) appears to decrease to a greater degree than muscle ACSA (approximately 2.3% to 2.7%/wk)[28,31].

Summary

Bed-rest appears to have varying degrees of impact on the upper and lower body. After 14 d of 6 degrees head down bed-rest maximum voluntary force for plantar flexion was decreased (approximately 7.5%/wk) whilst no effect was observed on maximal voluntary force of

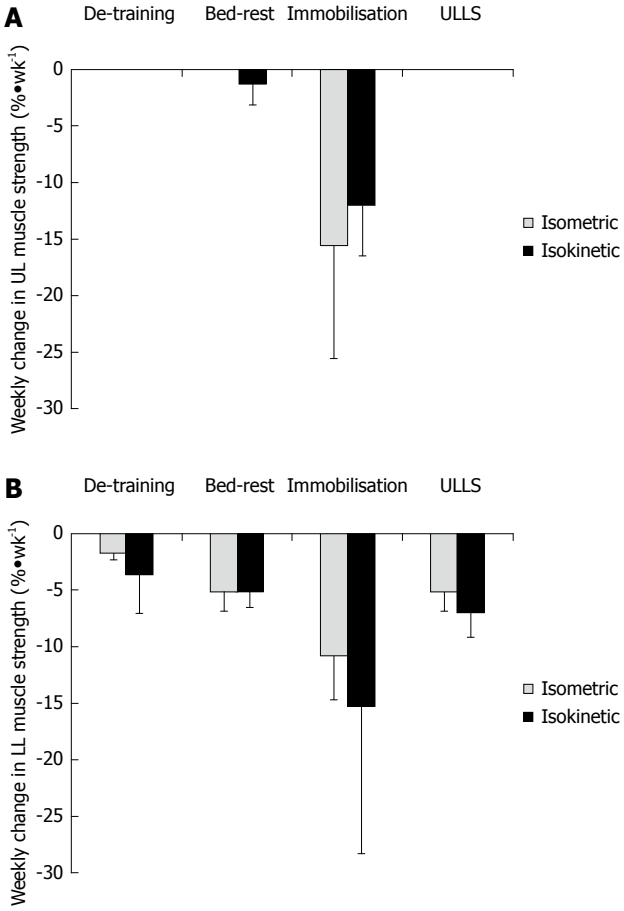

Figure 2 Relative change in isometric and isokinetic strength. A: Upper limb; B: Lower limb. ULLS: Unilateral lower limb suspension; UL: Upper limb; LL: Lower limb.

hand grip[52]. Similar results were demonstrated by LeBlanc *et al*[2] who showed after 17 wk of continuous bed-rest that isokinetic muscle strength decreased significantly in the thigh and calf with no loss in the arms. These results further support the idea that the lower limbs are primarily affected by bed-rest, more so than the upper limb. However, Gogia *et al*[63] did observe a decrease in elbow flexor torque (approximately 3.8%/wk) and a non-significant decrease in elbow extension torque (approximately 1.4%/wk) after 5 wk of bed-rest. Thus, suggesting that strength in the upper limb is affected by bed-rest but only in specific muscles during specific tasks.

Together, these findings show that in addition to the reduction in muscle mass, hypo-activity also results in a dramatic loss of strength [Figure 2 relative change in isometric and isokinetic strength in response to hypo-activity models. Figure 2A Values taken from references in the text for upper body changes in strength in response to de-training, bed-rest (isokinetic)[2,52,63], immobilisation (isometric)[12,23,56-58] (isokinetic)[23] and ULLS. Figure 2B values taken from references in the text for lower body changes in strength in response to de-training (isometric)[5,8] (isokinetic)[7], bed-rest (isometric)[1,52,53] (isokinetic)[18,53,59,60], immobilisation (isometric)[4,22,24,54,55] (isokinetic)[3,25] and ULLS (isometric)[28,29,31] (isokinetic)[30,61,62]. Where there are missing bars, this shows gaps in the

literature (*i.e.*, values are not available for that parameter during a specific hypo-activity models). Values are presented as means; error bars denote SD]. Models in which the joint is immobilised appear to have a greater impact on strength than unloaded models. These changes in muscular strength vary between hypo-activity models. The degree of loss in muscular strength surpasses the loss of muscle mass. Therefore, other alterations in the neuromuscular system, other than the reduction in contractile proteins must contribute to the excessive loss of strength. Voluntary force production is associated with neurological and skeletal muscle properties, thus suggesting these two factors as mechanisms accounting for the loss of strength with hypo-activity models.

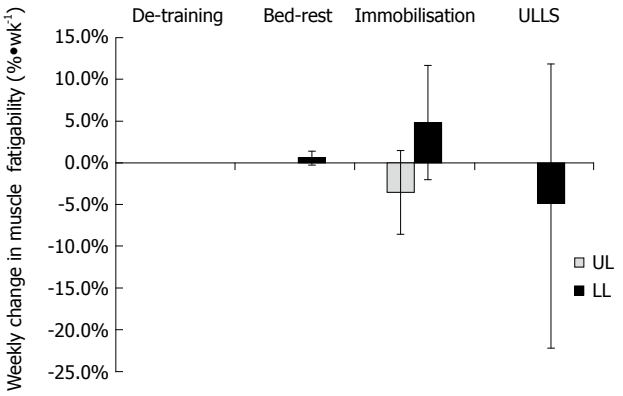

Figure 3 Relative change in muscle fatigability. ULLS: Unilateral lower limb suspension; UL: Upper limb; LL: Lower limb.

Muscle fatigability

Studies have also examined the impact of hypo-activity models on the fatigability of skeletal muscle. Kamiya *et al*[64] showed no change in time to fatigue after 14 d bed-rest. After a longer period of bed-rest (8 wk), Mulder *et al*[65] demonstrated an increase in fatigability (7.2%-10.2%/min decrease in maximum voluntary isometric torque per minute exercise; or approximately 0.9%-1.3%/wk fatigability increment). The contrast between the two studies would tend to suggest a delay in the impact of hypo-activity on muscle fatigability.

The effect of immobilising a limb has various different effects on skeletal muscle fatigability. Two weeks of full leg cast immobilisation resulted in no effect on muscle fatigability[4]. In contrast, Veldhuizen *et al*[3] found a decrease in isokinetic quadriceps endurance work from 9.1 kJ to 5.6 kJ after 4 wk leg cast immobilisation. These results suggest that short periods of lower limb immobilisation ($\leqslant$ 2 wk) have little effect on muscle fatigability whilst longer periods of immobilisation ($\geqslant$ 4 wk) increases muscle fatigability. Studies investigating the effects of immobilisation on skeletal muscle fatigability in the upper limbs have found different effects to those in the lower limbs. Similar to lower limbs shorter periods of immobilisation in the upper limbs appear to have minimal effects on muscle fatigability[23]. Unlike the lower limb, longer periods of immobilisation of the upper limb show a trend towards increased resistance to fatigability. Following 3 wk of hand-forearm immobilisation time to task failure increased by 21% (approximately 7%/wk)[66]. Semmler *et al*[56] investigated the effects of fiberglass cast immobilisation of the elbow joint, and reported 7 out of the 12 immobilised participants exhibited an unusual pattern of muscle activity during a fatiguing contraction after immobilisation. In those individuals with this unusual pattern of muscle activity there was an associated increase in the ability to maintain a contraction over an extended period of time in the elbow flexor muscles[56]. The physiological basis for the sometimes observed immobilisation-induced decreased fatigability, is not clear but it is likely to be related to neural factors[56]. In contrast to this, Miles *et al*[67] found an increase in fatigability in response to 3 wk arm suspension in untrained but not trained individuals. Previous research showed that ULLS led to increased fatigability after 4 wk of unloading[61]. Results from Deschenes *et al*[62] found a contrasting decrease in fatigability after just 2 wk of unloading.

Collectively these results suggest that muscle fatigability varies between different hypo-activity models [Figure 3 relative change in muscle fatigability in response to hypo-activity models (mean ± SD). Positive percentage change depicts an increase in fatigability whilst negative percentage change shows a decrease in fatigability. Values are separated into the effect of each hypo-activity model on the upper limb (UL) *vs* the lower limb (LL). The values are taken from the references used in the text for bed-rest (LL)[64,65], immobilisation (UL)[23,66] (LL)[3,4] and ULLS (LL)[61,62]. Where there are missing bars, this shows gaps in the literature (*i.e.*, values are not available for a parameter during a specific hypo-activity model)]. Shorter periods of hypo-activity ($\leqslant$ 2 wk) generally appear to have little impact on fatigability. Muscle fatigability appears to increase in weight-bearing muscles but immobilisation in the upper body suggests an increase in resistance to fatigue. Differences between studies could be due to the duration of unloading or in the method used to test fatigue resistance. The mechanisms that cause fatigue are specific to the task being performed[68,69]. Therefore, variability between fatigue resistance responses to hypo-activity models may be due to task specificity. Studies investigating a comparison of different fatigue tasks before and after hypo-activity are sparse. Yue *et al*[12] demonstrated a task-dependent effect on muscle fatigue with substantially increased endurance time (reduced fatigability) at a low force (20% MVC) and no statistical effect at a moderate force (65% MVC) in the elbow flexors. The selective improvement of fatigue resistance for the low-force contraction was accompanied by the absence of a change in the time course of the twitch, suggesting that the immobilisation-induced adaptation included and improved efficacy of some excitation-contraction processes and underscored the major role of these mechanisms in determining the endurance time for low-force, long-duration contractions. It appears that the hypo-

activity induced adaptations in muscle fatigability vary with the specifics of the task being performed. More research is needed to investigate these task-specific responses to different models of hypo-activity.

Numerous adaptations in fatigue mechanisms have been hypothesised to explain the observed preservation and decrease in fatigability in response to hypo-activity. As stated previously, hypo-activity results in muscle atrophy and a decrease in muscle strength, have been reported to be accompanied by myofiber transitions from slow to fast[70] and a shift in fuel metabolism away from lipid fuels toward glycolysis[71]. Typically these changes are associated with increased fatigability. Cardiovascular adaptations with hypo-activity[72] reduces oxygen delivery and oxygen utilization which may impair prolonged exercise capacity. Additionally, exercise tolerance may be influenced by impaired muscle activation after hypo-activity[1,54]. In light of this, the reports of decreased fatigability with hypo-activity are puzzling, and the underlying mechanisms remain unclear. It is possible that an atrophy-induced decrease in absolute force production will result in decreased intramuscular pressure. This in turn, will increase blood flow to the muscle and increase supply to match the metabolic demand[56,73]. Other potential mechanisms include adaptations in the neural activation strategy utilised[56], adaptations in the basal inorganic phosphate concentration[74], and changes in excitation-contraction coupling[12].

NUTRITIONAL SUPPLEMENTATION

As mentioned above, there is strong evidence that protein synthesis is decreased in response to periods of bed-rest and immobilisation[40,41,43]. That resistance exercise provides an anabolic stimulus during hypo-activity is undisputed[9,59,75]. When supplemented with nutritional interventions, the benefits of exercise during bed-rest appear additive[76], thereby suggesting different synergistic pathways for counteracting atrophy. It may not always be practical to prescribe exercise to counteract the atrophy brought about by inactivity. In these cases, such as trauma, pharmaceuticals may be used and have been tried with varying degrees of success[77]. However, effective long-term medication is not a palatable option (*e.g.*, costs, side effects, repeated injections). Where exercise is not a practical prescription, supplementing the diet with potential/recognised hypertrophic nutrients may be an effective and easily adhered to intervention programme for preventing the loss of muscle mass/function seen with hypo-activity. In this latter therapeutic group, potential candidates include proteins (essential amino acids (EAAs) and Leucine in particular), creatine, omega-3 fatty acids, vitamin-D (Vit-D) and antioxidants, to name but a few[78,79].

Protein

Stuart *et al*[80] sought to determine whether the catabolic effects of bed-rest in humans was due to a decrease in protein synthesis, and if so, to assess whether increasing the amount of dietary protein might be beneficial *i.e.* The calculated non-oxidative Leucine disappearance was used as a measure of whole-body-protein synthesis, which was shown to decrease when dietary protein was low. Bed-rest resulted in a 24% decrease in nonoxidative Leucine disappearance in participants assigned to a lower-protein diet (0.6 g protein·kg body $wt^{-1}\cdot d^{-1}$), whereas Leucine kinetics were unchanged by the same bed-rest protocol in participants who received a higher-protein diet (1.0 g protein·kg body $wt^{-1}\cdot d^{-1}$)[80]. In other words, whereas protein synthesis is suggested here to decrease with bed-rest, dietary supplementation of protein appears to protect against this deleterious response.

Essential amino acids

Bolus oral ingestion of EAAs produces a several-fold increase in plasma amino acid levels[81] and has been shown to stimulate net protein synthesis to a greater extent than a mixed meal or a solution containing nonessential amino acids[82]. Studies have shown that providing a nutritional supplement enriched with EAAs could improve lean body mass, strength and physical function even without exercise[83]. Previous studies by Stein *et al*[84,85] have shown improved nitrogen balance during both 6 and 14 d of bed-rest when provided with a daily supplementation of 11 g of branch-chain amino acids (BCAA), compared with the same dose of nonessential amino acids. It appears that a greater dose of EAAs (49.5 g/d) during 28 d bed-rest prevented any noticeable changes in muscle mass[86]. Paddon-Jones *et al*[86] however, reported that during this 28 d period that although no changes in muscle mass were observed they did find a decline in muscle strength. Nonetheless, the decrease in muscle strength with EAAs (11%) was still noticeably less than the decrease in strength seen in the control group (23%)[86]. These results collectively demonstrate a positive effect of EAAs supplementation during periods of bed-rest ranging from 6 to 28 d on both muscle mass and function[84-86].

Creatine

Creatine supplementation is another potential supplement that may attenuate hypo-activity induced decreases in muscle size and strength. Johnston *et al*[87] reported that short-term (29 d) creatine supplementation (20 g/d) attenuates the loss in muscle mass and strength during upper arm immobilisation. It is well known that muscle total creatine content can be rapidly raised by a high-dose oral creatine intake[88] and that long-term creatine intake can enhance the effects of weight training on muscle size and strength[89,90]. Creatine supplementation during 10 wk of resistance training has been shown to accelerate the rate of muscle hypertrophy in young adults who previously had their knee flexors immobilised for 2 wk[91]. Furthermore, 14 d creatine supplementation during hind-limb immobilisation lessened the rate of loss in the plantarflexors in a rodent model[92]. Additionally, Op't Eijinde *et al*[93] showed that creatine supplemen-

tation prevented the loss of glucose transporter type 4 (GLUT4) during muscle disuse and increased muscle GLUT4 content above normal levels during subsequent rehabilitation. Collectively these studies suggest that creatine supplementation during resistance training and rest may be effective at reversing or maintaining lower-body muscle mass during and after an immobilised state.

Antioxidants

Intricate antioxidant defence systems in the body work to continually manage oxidative stress. To counteract ROS, enzymatic and nonenzymatic antioxidants work together[94]. Enzymes work to improve or maintain an antioxidant balance and to avert oxidative damage by scavenging or preventing transformation of ROS to intracellular molecules and inhibiting their conversion to more deleterious forms. Endogenous nonenzymatic antioxidants such as vitamins-C and -E, carotenoids and flavonoids play important roles by contributing to the antioxidant system as cofactors for antioxidant enzymes. Results from Zwart *et al*[95] provide evidence that increased oxidative stress occurs during bed-rest. These data are also supported by results of several other studies that show evidence for elevated oxidative stress and increased ROS[96-98]. It would be interesting to see whether antioxidant supplementation during hypo-activity models will have beneficial effects on these outcome measures and furthermore, see whether this would then result in the attenuation of muscle loss in these models.

Vitamin-D

Ceglia proposed Vit-D supplementation as an effective nutritional intervention to attenuate age related sarcopenia[99]. Vit-D supplementation (800 IU per day) for periods of 8 to 12 wk has been reported to reduce postural sway and improve the risk of falling in elderly individuals[100,101]. Longer periods (12 mo) of Vit-D supplementation (800 IU per day) in the elderly has been shown to increase strength, decrease body sway and increase physical performance[102]. However, in a healthy elderly population with no Vit-D deficiency Vit-D supplementation does not appear to improve muscle strength or function[103,104]. It remains to be seen whether Vit-D supplementation in healthy persons with no Vit-D deficiency, any enhancement in muscle structural or contractile properties can be attained in the presence of hypo-activity.

Omega-3 (EPA)

Recent studies by Smith *et al*[105,106] supplemented healthy young and elderly individuals with omega-3 fatty fish-oils for 8 wk and found a significant increase in the muscle protein synthetic response to amino acid administration. They concluded in the elderly model that omega-3 fatty acids might be useful for the prevention and treatment of sarcopenia[105]. Dietary fish oil has also been shown to alleviate soleus muscle atrophy during immobilisation in association with Akt signalling in rats[107]. It would therefore seem reasonable to suggest that more investigation is needed into the potential of omega-3 fatty acids as a nutritional supplement for attenuating muscle atrophy with hypo-activity. In parallel, it is believed that omega-3 fatty acids may impact on lean body mass though decreasing the effectiveness of catabolic cytokines, reduced protein degradation and improving insulin sensitivity[108]. There is evidence to suggest that eicosapentaenoic acid (EPA) an omega-3 fatty acid may reduce the pro-inflammatory cytokines associated with inflammation[109]. Magee *et al*[109] demonstrated *in vitro* that EPA inhibits the effects of TNF-α by reducing its apoptotic effects and enabling myogenesis. It is however debatable whether this supplement would be useful in combating muscle atrophy where, as seen in human hypo-activity models, there is scant evidence for increased protein breakdown[40].

CONCLUSION

Hypo-activity models result in profound changes in skeletal muscle morphology and strength. Muscle mass and strength losses vary between different hypo-activity models, with immobilisation causing the most profound decreases, greater than bed-rest and limb suspension. Decrements in muscle size and strength are seen in response to hypo-activity models with the greatest decrements seen in antigravity muscles. The decreases in strength seen with hypo-activity models surpass the losses in muscle mass and as such, the nervous system and contractile properties adapt to adjust for this excessive loss of strength. Nutritional supplementation may stand as a viable intervention to combat muscle atrophy with hypo-activity when exercise is not a practical prescription. There are several potential nutritional supplements that could be used to combat muscle atrophy but extensive research is needed to determine the most affective.

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P- Reviewer: Gorgey AS **S- Editor**: Song XX
L- Editor: A **E- Editor:** Liu XM

World Journal of Translational Medicine

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.5528/wjtm.v2.i3.49

World J Transl Med 2013 December 12; 2(3): 49-55
ISSN 2220-6132 (online)

REVIEW

Impact of viral and bacterial infections in coronary artery disease patients

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Received: August 16, 2013 Revised: September 30, 2013
Accepted: November 1, 2013
Published online: December 12, 2013

Abstract

Atherosclerosis is becoming an alarming disease for the existence of healthy human beings in the 21st century. There are a growing number of agents, either modernized life style generated, competitive work culture related or infection with some bacterial or viral agents, documented every year. These infectious agents do not have proper diagnostics or detection availability in many poor and developing countries. Hence, as active medical researchers, we summarize some aspects of infectious agents and their related mechanisms in this review which may be beneficial for new beginners in this field and update awareness in the field of cardiovascular biology.

Key words: *Chlamydia pneumoniae*; *Helicobacter pylori*; *Cytomegalovirus*; Cytokines; Diagnostics

Core tip: This paper describes the association of atherosclerosis with different infectious agents, specifically *Chlamydia pneumoniae* (*C. pneumoniae*), *Helicobacter pylori*, Herpes viruses and periodontal pathogens. There are many other bacteria and viruses, as well as life style related factors described and cited in this review. The manuscript also emphasizes how *C. pneumoniae* is modulating the human immune system with mimicking some antigenic proteins of the host. Overall, this report helps in the field of cardiac biology to explore associated risk factors in more detail.

Jha HC, Mittal A. Impact of viral and bacterial infections in coronary artery disease patients. *World J Transl Med* 2013; 2(3): 49-55 Available from: URL: http://www.wjgnet.com/2220-6132/full/v2/i3/49.htm DOI: http://dx.doi.org/10.5528/wjtm.v2.i3.49

INTRODUCTION

There are numerous studies supporting the association of coronary artery disease with many infectious agents, including bacteria and viruses[1-8]. Several bacterial pathogens have been reported to trigger the inflammation of atherosclerosis, including *Chlamydia pneumoniae* (*C. pneumoniae*)[7,9,10], *Helicobacter pylori* (*H. pylori*)[11,12], *Chryseomonas sp*[13], *Veillonella sp*[13], *Streptococcus sp*[13], *Aggregatibacter actinomycetemcomitans*[14], *Porphyromonas gingivalis*[15], *Prevotella intermedia*[16], *Prevotella nigrescens*[17], *Tannerella forsythia*[18], *Ruminococcus enterotype*[19], *Enterobacter hormaechei*[20] and periodontal pathogens[21]. Similarly, many viruses are known to be associated with atherosclerosis, namely *cytomegalovirus* (CMV)[22,23], *herpesvirus*[24], *hepatitis A*[25], B[26] and C viruses[27], *Epstein-Barr virus*[28] and *Herpes simplex virus* I and II[29]. Thus, it would be important to know in which circumstances bacterial and viral infections activate heart disease mechanistically.

REVIEW OF THE LITERATURE

Increasing the risk of heart disease is a major cause of concern. In 2008, 30% of all global death was attributed to cardiovascular diseases[30]. It is also estimated that by 2030, over 23 million people will die from cardiovascular

diseases annually[30]. The incidence rate of atherosclerotic symptoms is increasing exponentially year by year[31]. There are numerous factors involved in the causation of atherosclerosis. Some researchers strongly classify it as a life style disease, including body mass weight, smoking, heavy alcohol intake, sedentary life style, blood pressure, elevated levels of cholesterol and bad lipids, reduced levels of good lipids and a stressful life[32-38]. Many studies have found a significant association of atherosclerosis with genetics or as hereditary[39], with close blood relatives suffering from heart attack, diabetes or hypertension[8,40,41]. Moreover, mainly from last decades, various studies were conducted on the association of heart disease with infectious agents. Many types of specimens, including blood samples, PBMCs and specific tissue sites were evaluated for the establishment of infection with atherosclerosis[42]. To date, there are hundreds of research studies using ELISA, standard PCR, real time quantitative PCR, cell culture, immunohistochemistry and immunocytochemistry methods to find a relevant and authentic answer for the association between infectious agents with atherosclerosis[6-8,43-49]. Although some controversy exists in this field in order to completely accept the direct association between infectious agents with atherosclerosis, there is no question of the enhanced presence of infectious agents in atherosclerosis or accelerated progression of atherosclerosis in the presence of infectious agents. To date, some well established infectious agents, like bacteria and viruses, *C. pneumoniae*, *H. pylori* and *cytomegalovirus,* were observed in a number of studies and explained the etiology of disease causation in detail[50-53].

C. PNEUMONIAE

C. pneumoniae is an intracellular obligatory bacteria which causes upper and lower respiratory tract infections[54]. Other than respiratory disease, *C. pneumoniae* has been found to be associated with heart disease, Alzheimer's disease, multiple sclerosis, lung cancer and arthritis[55-59]. 95% of the population is exposed to *C. pneumoniae* in their life time; however, this exposure is asymptomatic while in contact with *C. pneumoniae* frequently and exposure to some other co-activator of *C. pneumoniae* infection triggers the establishment of infection and chronicity of disease pathogenesis[60]. There are numerous tissue or body organelles involved in the acceleration of *C. pneumoniae* infection[61,62]. Correct diagnosis of infectious agents is always in question and many methodological improvements have been made in this aspect[63,64]. To date, nested PCR or quantitative probe based real time PCR methods have been largely updated in this field[7,65,66]. 16S rRNA and major outer membrane protein have been found to be critical for identification on PCR based methods[7,67,68]. Moreover, immunoglobulin based screening also has significance and capability for the predication of disease occurrence in existing non-symptomatic and close relative populations of patients[41]. In many studies, *C. pneumoniae* specific immunoglobulin IgA has been found to be more predictive and robustly observed compared to IgG in the serum of coronary artery disease patients[8,69,70], while some studies reported it vice versa as well[71]. In response to *C. pneumoniae* infection, many host immune responses are manipulated or aggravated to counter the effect of bacterial pathogens and stop the progression of disease, while at the same time, this smart bacteria also activates host signaling by mimicking some of the key proteins, starting to accelerate disease progression[49,72,73]. These host-pathogen responses are very complex and many studies find some narrative result which suggests the hypothetical model for the infection progression due to *C. pneumoniae*[74]. Moreover, details are needed to explore this field to prevent infection of the human population from these kinds of opportunistic pathogens.

H. PYLORI

H. pylori is known to be an active initiator of gastric carcinoma[75]. Moreover, the presence of *H. pylori* has been found to be associated significantly in atheromatous plaque[76]. In our study, we found significant *H. pylori* IgA antibody titer in CAD patients compared to controls and levels of H. pylori IgG were also high[8]. Furthermore, we also detected *H. pylori* DNA in atheromatous plaque by using quantitative real time PCR[6]. There are many other reports also suggesting the active involvement of H. pylori in the development of atherosclerosis[11,77,78]. However, it is important to know in which circumstances this bacterium activates oncogenesis and heart disease.

CMV

CMV is an important pathogenic virus which causes many chronic diseases, such as cancer and atherosclerosis[79-82]. There is growing evidence supporting the synergistic effect of infectious agents in the progression of heart disease[50,83]. In our antibody titer detection assay and PCR assay, we found higher positivity for CMV in CAD patients compared to controls[6]. However, there is lots of space where we can identify the initiator organism or activator organism among many infections which may alter the immune response of systems.

HUMAN HERPES VIRUSES

Evidence suggests that human herpes viruses have a potential link to arterial injury[83]. This hypothesis is proven in animal model studies, as well as a clinical epidemiological association between herpes viral infection and accelerated arteriosclerosis[84]. Studies suggested that eight members of the herpes virus family member may infect humans[85]. *Herpes simplex virus*-1 (HSV-1), *herpes simplex virus*-2 (HSV-2), *Epstein-Barr virus* (EBV) and CMV are widespread in the general population; they are primary candidates for investigations into viruses related to ath-

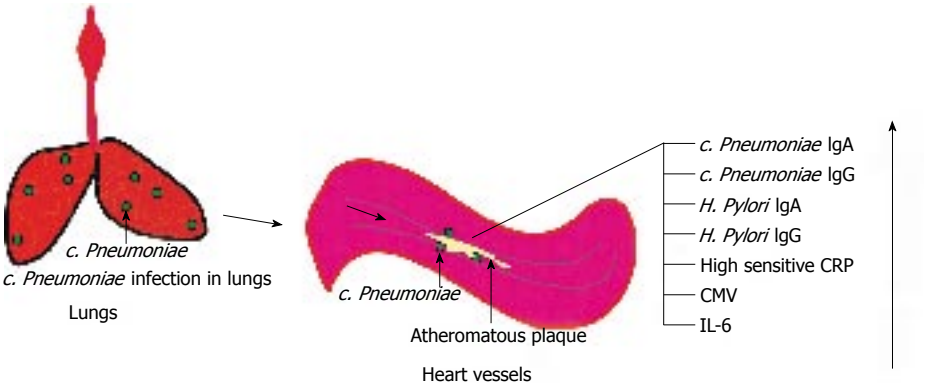

Figure 1 A schematic representation of *Chlamydia pneumoniae* infection from lungs to heart. IL: Interleukin-6; CMV: *Cytomegalovirus*; CRP: C-reactive protein; *c. Pneumoniae*: *Chlamydia pneumoniae*; *H. Pylori*: *Helicobacter pylori*.

erosclerosis[86].

A definite association was found for HSV-2 and subclinical coronary atherosclerosis[87]. This organism has been shown to be responsible for thrombogenic and atherogenic changes to host cells[88]. Earlier association of HSV-2 with hypertension has been reported[89]. These days, many studies emphasize the role of inflammatory pathways in atherosclerosis development[90]. Furthermore, recently Horváth *et al*[91] suggested that long-term HSV-2 infection may contribute to the development of atherosclerosis.

Many earlier studies demonstrated that only atherosclerotic tissues majorly have multiple infections[86]. Researchers also suggested that the synergistic impact of infection on atherogenesis is related to the aggregate number of pathogens infecting human beings[92]. Several serological studies demonstrated that all these pathogens (CMV, EBV, *hepatitis A virus*, HSV-1, HSV-2 and *C. pneumoniae*) are variably associated with the risk of CAD[4]. Shi *et al*[86] detected HSV-1, EBV and CMV DNA in the upper part of the non-atherosclerotic aortic wall and these viral DNA were also detected more extensively in atherosclerotic lesions compared to non-atherosclerotic tissue.

DENTAL PATHOGENS IN ATHEROSCLEROSIS

There are several reports with an emphasis on the association of dental disease with elevated risk of myocardial infarction[93] and metabolic activity of the gut microbiota has also been shown to be related to blood pressure[94]. Several other studies also suggested an oral source for atherosclerotic plaque-associated bacteria[95,96]. *Chryseomonas sp* was present in endocarditis and all atherosclerotic plaque samples[97].

Many species, namely *Porphyromonas gingivalis*, *Tannerella forsythia* and *Actinobacillus actinomycetemcomitans,* are actively involved in periodontal disease and have been reported as a potential risk for the development of atherosclerosis[98]. Animal studies have also proven this association[99].

Beside these infectious agents, other factors that may incite vessel inflammation are oxidized low-density lipoprotein cholesterol and the metabolic syndrome, which are associated with a proinflammatory condition characterized by elevations of C-reactive protein or high sensitive C-reactive protein (hs-CRP)[100-102]. Metabolic syndrome is a cluster of abnormalities caused by elevation of multiple metabolic pathways, hyperinsulinemia, insulin resistance in body organelles, hyperglycemia, atherogenic dyslipidemia, abdominal obesity and hypertension[103-104]. In our study, we found the association of hs-CRP with elevated levels of *C. pneumoniae* IgA and H. pylori IgA[8]. We also observed higher proinflammatory cytokines interleukin-6 positively associated with hs-CRP[100]. Furthermore, our study extended the knowledge in respect of the association between C. pneumoniae IgA with Th-1, Th-2, Th-3 or adhesion molecules[101], although these markers were labeled as independent markers for CAD in our study[105]. There are many studies that suggest that Th-1 cytokines or proinflammatory cytokines are expressed earlier after *C. pneumoniae* infection followed by Th-2 kind of cytokines[106-107]; however mechanistically it moves in the case of humans is still evaded. We draw a schematic for *C. pneumoniae* in atherosclerosis (Figure 1).

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P- Reviewers: Han Q, Niculescu M **S- Editor**: Song XX
L- Editor: Roemmele A **E- Editor:** Liu XM

World Journal of Translational Medicine

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.5528/wjtm.v2.i3.56

World J Transl Med 2013 December; 2(3): 56-66
ISSN 2220-6132 (online)

REVIEW

Concepts of body constitution, health and sub-health from traditional Chinese medicine perspective

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Received: June 4, 2013 Revised: July 27, 2013
Accepted: August 17, 2013
Published online: December 12, 2013

Abstract

This paper described and discussed the important literature and ideas about the concepts, types and measurement of body constitution, in terms of healthy, sub-healthy and disease status. In view of traditional Chinese medicine, "healthy" state is a status of relative balance of Yin and Yang to keep our bodily homeostasis. If there are significant physical and/or psychological stressors, such as loss of a beloved one and failure in study or work, the body can no longer keep its own bodily condition balanced and subsequently enter a state of "sub-health" (sub-optimal health). "unhealthy" body constitution such as "Dampness-heat", "Cold-dampness" and "Heat- or Cold- dryness" with a subnormal body temperature and humidity and clinical manifestations such as insomnia, malaise and overweight will be presented. Immediate, appropriate strategies such as modification of life-style and seeking medical treatment can prevent evolution of an illness. Otherwise, the body will enter a disease status with a "pathological" body constitution of "Yin or Yang deficiency", "Blood-stasis" and/or "Phlegm-dampness". To be complimentary with health promotion and disease prevention in Western medicine, understanding about an individual's body constitution, together with its determinants (*e.g.*, healthy eating and lifestyle behaviors), can contribute to a more proactive, holistic and individualized healthcare.

Key words: Body constitution; Health; Sub-health; Disease; Traditional Chinese medicine

Core tip: This article discussed the concepts of body constitution in traditional Chinese medicine, which can reveal and advocate an individual's bodily condition and functioning and thus contribute to a more proactive, holistic and individualized healthcare. We critically discussed the concepts, types and measurements of body constitution in terms of three main health statuses - healthy, sub-healthy and disease. With better categorized "healthy", "sub-healthy" and "unhealthy" patterns of one's body constitution, the levels of bodily resistance (strong or weak) and functioning of internal organs (adaptive or mal-adaptive), as well as the balance of "Yin and Yang", can be easily differentiated and maintained.

Chan RYP, Chien WT. Concepts of body constitution, health and sub-health from traditional Chinese medicine. *World J Transl Med* 2013; 2(3): 56-66 Available from: URL: http://www.wjgnet.com/2220-6132/full/v2/i3/56.htm DOI: http://dx.doi.org/10.5528/wjtm.v2.i3.56

INTRODUCTION

Chronic diseases such as cancer, arthritis, dementia and stroke impose wide varieties of medical and health problems and poor quality of life[1] and even take away a lot of people's lives every year. As a result, these diseases can cause huge burden to the health care costs across countries[2]. In addition, it is also estimated that the aged popu-

lation (65 years old or above) will increase from about 8% in 2010 to 16% in 2050[3]. As such, the incidence and prevalence of chronic diseases, especially in the middle age and older people, will continue up-surging. Besides disease prevention, prophylactic intervention such as healthy eating and lifestyle and early seeking medical advices and treatments can be considered in health promotion and/or interfering the disease development during the pre-morbid stage.

"Sub-health" is commonly used to describe the condition of pre-morbid or prodromal stage of health problems among health professionals in mainland China. The concept of "sub-health" was developed on the basis of the "third state of health" first introduced by Professor Buhemann in the Soviet Union[4], to describe there is a sub-optimal health status in-between the healthy and disease condition. People who have subjective complaints about early symptoms and discomfort may not receive a confirmed medical diagnosis by the physician (Western medicine) because of the negative or unclear results of medical or pathological investigations. According to a survey conducted in 2006 on 3624 people in China, more than 75% was at the state of sub-health[5]. The researchers suggested that majority of these people might soon proceed to certain kinds of medical disease, if not treated or intervened. However, many people may feel unacceptable, unbelievable and helpless at the time of receiving a medical diagnosis of one life-threatening disease; and sometimes, they may feel regretful for not having taken care of themselves or their beloved one properly. It is therefore questionable whether we can intervene on the sub-healthy condition with prophylactic treatments before it is confirmed with a medical diagnosis.

Traditional Chinese medicine (TCM) advocates "treating an illness before it happens"[6], that is to give intervention or treatment according to one's body constitution whenever it shows changes in patterns and thus appears to be unhealthy. The term "body constitution" (BC) is commonly used in Chinese medicinal literature and culture to describe one's current health status, reflecting his/her bodily condition and functioning[7]. People are empowered to take care of themselves with lifestyle modifications and/or proper dietary practices, according to their BC prior to any illness occurrence, and thus crucial to "promote health, prevent disease and enhance longevity". TCM practitioners give individualized treatment to each client according to his/her BC[8] and treat disease "from its root" (*i.e.,* the causes or rationale behind the presenting signs and symptoms)[9]. Therefore, knowing one's own BC is one of the most important steps to promote his/her health condition, as well as his/her quality of life.

In recent 30 years, TCM practitioners and researchers have put much effort to develop a clear concept of BC. But till now vigorous debates and discussions are persisted on several important topics, including mainly the definition and types of BC, categorization of BC by using the common medical terms of TCM, and symptom differentiation in terms of different types of BC[10]. In views of the complexity of the concept of and different beliefs and perspectives toward BC, this paper is to describe and discuss the important concepts and views of BC in terms of health, sub-health and disease conditions in order to increase our understanding and reveal the significance of BC to the knowledge of TCM, as well as our health and well-being.

CONCEPT OF BODY CONSTITUTION AND ITS MEASUREMENT

Starting from the ancient time, people have been very interested in knowing the commonalities and differences on characteristics and features of human beings, between individuals and/or among ethnic groups/flora. Interestingly, even though different perspectives or systems between the Western and Eastern parts of the world, their central themes or concepts of classification of biological features in humans or animals are very similar. In general, people are classified into groups based on their morphology, which is similar to elephant or deer[11], or on a whole picture of physiological and psychological conditions such as in terms of "four body fluids" introduced by Hippocrates, including sanguine, phlegmatic, bilious, and melancholic[12]. This belief is compatible with the principles of TCM in understanding about the "types, shapes and states" of a human body in relation to BC stated in the Yellow Emperor's Canon of Medicine[11]. According to the "Yin-Yang" theory, different body appearances and personalities can be classified into "25 Types of People" in terms of their skin color, body shape, personality, and adaptation to natural environment and climate change[11].

Not until the Hon Dynasty, a famous TCM scholar Cheung Chung-king indicated that the body nature or condition was related to the disease occurrence, development, treatment and prognosis. He suggested that different kinds and severity of symptoms could co-exist in the course of the illness for different people even when they suffered from the same disease, or received the same medical diagnosis. This may be due to the differences on their individual body conditions, which can be categorized people into different types of body characteristics such as strong, weak, ulcer, and bleeding types, regarding one's risk or tendency of disease development[13]. Cheung's suggestions had nourished the concept of BC and researchers started to adopt TCM terms in the classification of BC in the late 19th century. A few TCM experts in China, especially Professor Kuang DY and Professor Wang Q, shared about their views and initiated in establishing the theoretical framework and concepts of BC.

Conceptually, BC represents the nature of the person's morphosis, physical and psychological components at one point of time, or sometimes a short period[14]. Although Kuang[15] argued that one's psychological status should be classified in terms of temperament, it is well known to be affected by both congenital and acquired factors, together with relative group tendency (in relation to their

environmental and cultural situations) and absolutely personalized bodily condition. Each person should possess a few characteristics similar to their ancestors such as skin and hair colors, bodily appearance and features and stature. Similarities and commonalities of basic bodily health and functioning may co-exist in a racial or an ethnic group, because they are likely to share common characteristics and backgrounds in relation to their living environment, race, eating behaviors and life habits, interest, and basic genetic manifestations[16]. For instance, a group of children or elderly people who are at the same developmental stage may possess similar bodily features and physiological characteristics earmarked by that particular human developmental stage. Interestingly, the BC of the closest family members such as parents, siblings and twins can be unique, while some diseases such as *Hepatitis B* carriage gene can be inherited from parents. In addition, body frame of the Western people are often bigger than that of the Eastern people because of their differences on genetic manifestation. Due to different combinations of personal, environmental, social, cultural and geographical factors, the BC of people can be absolutely different between each other. Parents' health, especially those mothers during pregnancy and even genetic changes or mutations, can impose much varied levels of impacts to the bodily condition of the next generation. For instance, congenital heart disease may be developed when fetus is growing in his/her mother's uterus[17], and the HIV infection can be transmitted during birth[18].

Despite one's BC being influenced by various external (*e.g.,* environment and climate) or internal (*e.g.,* hormonal or sudden changes in biological conditions) factors, it seems relatively stable due to the governance of family inheritance or genetics, race and sex, imposing lifelong effects to an individual's bodily condition. Throughout one's developmental life stages, BC changes progressively within and between each life mileage and, however, critically in a few essential life stages such as from children to adolescence[19]. But prolonged or significant internal and external factors may induce continuous changes, or capricious shifts in different lifestyle patterns or behaviors such as work[20], eating behaviors and living environment[21]. However, these effects to the BC may become settled, or constant, once the person can adapt well to the influencing factors, resulting from an effective buffering mechanism ("strong" in bodily condition) inside the human body[22,23]. But if the stress or its ill effects are prolonged, negative (maladaptive) and dysfunctional patterns of BC will appear.

Kuang[24] indicated that BC would initiate pathological changes within body during the transitional periods of pre-diagnosis (prodromal stage) and convalescence. Therefore, it is believed that BC may contribute to the level of susceptibility and clinical outcomes of a disease, presenting in various forms, characteristics (like signs and symptoms), or patterns of resistance and reactions towards the existing internal and external stimuli. Therefore, Kuang[24] also suggested that physical and psychosocial development and environmental changes of an individual can induce progressive changes to one's BC; and its obvious changes often occur during the life development milestones such as pregnancy, middle age and menopause[19].

In addition, shifts of types and features of BC will also be caused by both internal and external factors, including parents' health and inheritance, environmental stressful situations and emotional frustrations, exercise and eating habit[25], bodily disease and treatment[26]. Similarly, it is also believed that "unhealthy" BC can be adjusted by medications[27] and diet[28]. Modifications of parents' or mother's BC could indirectly improve, or jeopardize, the health status of their baby over the period of fertilization and/or pregnancy[29].

In order to understand one's own BC, on-going assessment by using the four classical diagnostic skills of TCM[30], including inspection (patient's facial colour, tongue sign and external appearance), listening (patient's voice and coughing sound) and smelling (patient's body odour), inquiry (patient's complaint) and palpation (patient's body part and pulse) and questioning are crucial.

Different measurement tools of BC can also be considered in TCM diagnosis and research. The 60 items Constitution in Chinese Medicine Questionnaire (CCMQ) is a widely used assessment tool in China developed by Zhu *et al*[31] in 2006 to measure nine types of BC, including Gentleness, Qi-deficiency, Yang-deficiency, Yin-deficiency, Phlegm-wetness, Wet-heat, Blood-stasis, Qi-depression and Special diathesis. The strength of the scale is the vigorous face and content validity done by the Chinese Medicinal experts. However, a few limitations are noted. First, only concurrent validity has been demonstrated with SF 36 and body mass index[32], and thus the sensitivity and specificity of the instrument is in doubt. In principle, healthy BC should not be identified in patients with disease. Interestingly, "Gentleness" (healthy) type of BC was identified in ill persons, using this CCMQ, such as 37.4% of 147 subjects with liver cancer[33] and 43.6% of 101 subjects with hypertension[34]. Another concern is the use of retrospective data that examined on the signs of illness within one year is considered unreliable due to the result bias and difficulty in reporting those repeatedly occurring items. Wang *et al*[35] criticized that the nine subscale (*i.e.,* nine types of BC) of the CCMQ was confusing, non-specific in categorizing BC types, and indicating inconsistent of results by using both the Chinese Medicinal terms in pattern differentiation and the allergic reaction in Western Medicine (especially for the "Special diathesis" subscale). The specificity of the instruments are questionable in assessment of BC.

Ho *et al*[36] in 1996 developed a 96-item Chinese Construction Questionnaire (CCQ) by clustering analysis of > 5000 sets of data from people in different provinces of China to identify six types of BC, including "Strong", "Weak", "Trend-Cold", "Trend-Heat", "Trend-Dampness", and "Blood-stasis". This assessment scale is flexible in detecting types of BC; clustering analysis can be

used in order to identify additional types of BC based on the six basic types mentioned above. Researchers identified eight types of BC in 809 hepatitis B patients[37], and 16 types in 879 obesity people[38] by using the CCQ. As the questionnaire is too lengthy and with limited evidence on reliability and validity, it has not yet been widely used.

In Taiwan, Su[39] in 2008 developed a self-rating 22-item BCQ+: A Body Constitution Questionnaire to assess Yang-Xu (*i.e.,* the energy levels of different bodily functions in terms of five internal viscera). The questionnaire is easily administered and indicated satisfactory psychometric properties such as content validity and internal consistency. Nevertheless, it only focuses on a single dimension of bodily condition (Yang-Xu) and thus shows incomplete picture of BC. Similarly, several scales measuring single type of BC such as the Yin-deficiency Questionnaire[40], Yin Scores and Yang Scores[41], and Cold-Heat Pattern Questionnaire[42], Phlegm Pattern Questionnaire[43], and Kidney deficiency Syndrome questionnaire (KDSQ)[44] have been found for TCM diagnosis. There are also a few measurement tools of health condition using the concept of BC, such as the Health Scale of Traditional Chinese Medicine[45] assessing health perception and Chinese Quality of Life Instrument[46] assessing the health-related quality of life.

In conclusion, BC not only can describe and represent the current bodily condition of an individual, which is relatively stable over time, but also can reveal progressive changes and responses toward the internal and external stimulations in a person's body. A healthy person with strong and functional internal organs is able to better adapt and remain healthy if the overall stimulations and their bodily responses are below one's threshold of bodily resistance or satisfactorily coped with homeostasis mechanisms of the body. This is also compatible to the concept of "health and illness continuum" in Western medicine[47], in which health of every person condition is not constant, for instance, people may feel mild discomfort during daytime but then they feel better again spontaneously or after taking rest and self-regulatory actions such as taking a snap and drinking cups of water. However, for a vulnerable person with sub-normal function of the internal organ(s), or "sub-health", progressive pathological changes will occur and thus the person's health condition may shift from a normal or "healthy" to an "abnormal" BC.

Common types of BC

In general, two major types of BC, including normal and abnormal, can be categorized and considered to be more simplified and easily understood in describing one's bodily condition and functioning. While the term "normal" implied a healthy, positive and good, a "normal or healthy" BC should be pointed to an absolute healthy condition without any signs of disease and on the other hand, with strong and pleasant appearance and good functioning in all main body organs and systems such as nervous, cardiovascular and respiratory system, as well as being able to indicate "normal" results in various physical and psychological investigations[48]. A normal BC should also present with good hardiness and quality of life and having adequate ability of coping with stress, and high adaptability to external socio-economical and natural environments. On the other hand, an "abnormal" BC implied unhealthy, negative or poor bodily condition. Wang[8] adopted the term "biased" or "deviated" to describe such an abnormal condition, describing it as "sub-health" in association with specific type(s) of disease(s) or ill condition. However, most recent classifications of BC focused on the pathological changes of the bodily condition[10], thus increasing the use of more complex pattern differentiations to classify different types of BC.

In modern China, researchers based on the perspective of TCM practitioners to develop more than 60 taxonomies of BC[10]. As suggested by Kuang[24], while many practitioners may believe that BC were mainly abnormal relating to pathological changes, it is noteworthy that some have recognized a few types of BC to be "healthy" or "normal". The classifications of BC much varied, from two to 16 types. Recently, Kung[49] has summarized 19 major classifications of BC (see Table 1) developed between 1978 and 2002.

All of them were developed on the basis of the theories of TCM and clinical symptoms of diseases. One major commonality among these classifications is that they have adopted "patterns" (of features or signs) in the concept of TCM to explain the bodily condition. For instance, 10 of them considered the level of "fluid", "Qi" and "Blood", 9 adopted the theory of "Yin and Yang", 10 adopted "Phlegm", and 3 included "Hot" and "Cold". In addition, the other concepts included in the classifications like "Strong" and "Weak" (in 2 classifications), visceral functions (6 of them), and "normal" types of BC (15 of them). In recent years (2006-2013), most TCM researchers based on Wang's[67] nine types of BC (Gentleness, Qi-deficiency, Yang-deficiency, Yin-deficiency, Phlegm-dampness, Heat-dampness, Blood-stasis, Qi-depression, and Special diathesis) to look into different kinds of illnesses or diseases, in particular dementia[68], ischemic stroke[69], osteoporosis[70] and renal disease[71]. Based on Ho's[36] six basic types of BC, a few TCM researchers also identified eight types of BC (Normal, Qi-depression, Qi- deficiency, Stagnation, Yin-deficiency, Deficient-cold, Internal-heat , and Anxious) to assess health condition of people with hypertension[72] and coronary heart disease[73].

Another major classification of BC is based on the functioning of internal organs, commonly used in bronchitis asthma[74] and post-infection condition of children[75]. "Yin and Yang" types of BC have been used for assessing the BC concerning insomnia[76]; and Five Elements ("Metal", "Wood", "Fire", "Earth", and "Water") and "Cold"and "Heat" types are commonly used in examination and diagnosis of with metabolic disorder[77] and naso-pharyngeal carcinoma[78], respectively.

Though there are different types of classification of

Table 1 Classifications of body constitution (1978–2002)

Authors and number of types	Types of body constitution
So *et al*[50] (1996); 3 types	Balance type; Spleen-Lung type; and Spleen-Kidney type
Li *et al*[51] (1996); 3 types	Yang exuberance type; Yin exuberance type; and Yin and Yang harmony type
Hu *et al*[52] (1987); 4 types	Harmony type; Functional exuberance type; Functional deficiency type; and Functional exuberance and deficiency type
Zhu *et al*[53] (1989); 5 types	Normal type; Phlegm-dampness type; Qi-deficiency type; Internal-heat type; and Qi-Yin deficiency type
Wan *et al*[54] (1998); 5 types	Yin and Yang balance type; Heat-stagnation type; Spleen-Stomach Qi-deficiency type; Spleen-Stomach Yin-deficiency type; and Spleen-Stomach Qi-Yin-deficiency type
Kaung *et al*[55] (1978); 6 types	Normal type; Heat-dryness type; Blood-stasis type; Phlegm-wetness type; Yang-deficiency type; and Fatigue-pale type
Ho *et al*[56] (1986); 6 types	Normal type; Yin-deficiency type; Yang-deficiency type; Yin & Yang-deficiency type; Phlegm-wetness type; and Blood-stasis type
Ho *et al*[36] (1996); 6 types	Strong type; Weak type; Trend-cold type; Trend-heat type; Trend-dampness type; and Blood-stasis type
Pang *et al*[57] (1985); 7 types	Normal type; Excess-heat type; Qi-stagnation blood-stasis type; Phlegm-dampness type; Deficiency-cold type; Qi-blood deficiency type; and Yin-deficiency type
Qin *et al*[58] (1984); 7 types	Norma type; Yin-deficiency type; Yang-deficiency type; Phlegm-dampness type; Qi-deficiency type; Heat-dampness type; and Blood-stasis type
Wang *et al*[59] (1995); 7 types	Normal type; Spleen-insufficient type; Kidney-insufficient type; Lung-insufficient type; Liver-insufficient type; Heart-insufficient type; and Fetus-heat type
Chen *et al*[60] (1988); 7 types	Normal type; Yin-deficiency type; Yang-deficiency type; Kidney-deficiency type; Qi-Blood-deficiency type; Phlegm-dampness type; and Blood-stasis type
Chen *et al*[61] (1998); 7 types	Normal type; Yin-deficiency type; Yang-deficiency type; Phlegm-dampness type; Qi-blood-deficiency type; Fatigue type; and Yang- exuberance type
Wang *et al*[62] (2002); 7 types	Gentleness type; Yin-deficiency type; Yang-deficiency type; Qi-deficiency type; Blood-stasis type; Phlegm-dampness; and Heat-dampness type
Wang *et al*[63] (1984); 9 types	Yang type; Yin type; Yin-deficiency type; Yang-deficiency type; Heart-deficiency type; Liver-excess type; Spleen-deficiency type; Lung-deficiency type; and Kidney-deficiency type
Mu *et al*[64] (1983); 9 types	Qi-deficiency type; Blood-deficiency type; Phlegm-wetness type; Blood-stasis type; Yang-deficiency type; Yin-deficiency type; Yin-exuberance type; Yang-exuberance type; and Qi-stagnation type
Tian *et al*[65] (1983); 12 types	Yin-deficiency type; Yin-cold type; Yang-deficiency type; Yang-heat type; Qi-deficiency type; Qi-stagnation type; Blood-deficiency type; Blood-stasis type; Fluid-deficiency type; Phlegm-dampness type; Stirring-wind type; and Toxin type
Niu *et al*[66] (2001); 16 types	Harmony type; Anxious type; Unbalance type; Stagnation type; Interior heat type; Liver-stagnation type; Phlegm-dampness type; Blood-stasis type; Weakness type; Yang-deficiency type; Qi-deficiency type; Essence-depletion type; Fluid-depletion type; Lung-deficiency type; Spleen-deficiency type; and Heart-blood deficiency type

BC, we can conclude that all types of BC can be categorized according to "patterns", or contrasting (like opposite ends) features or states described in TCM, such as "Yin and Yang" (deficient or excess), "Blood" and "Qi" (stasis, deficiency or stagnation), functional excess or deficiency (Spleen, Heart, Lung, Kidney and Liver), and "Phlegm and Wetness", except a few "normal" BC. There are 15 out of 19 commonly used classifications included the "healthy" BC as one of the major types; and the rest classifies BC as various bodily conditions at risk of some kinds of diseases with manifestation of certain pathological changes. In addition to the four classical assessment skills in TCM (inspection, auscultation and olfaction, inquiry, and pulse taking and palpation) used, it is recommended that for the purpose of primary prevention and routine screening, user-friendly, efficient and appropriate approaches of BC assessment for the general public should be developed. In order to enhance self-evaluation of one's own BC, self-report valid questionnaires should be designed, whereas a few have been developed and obtained preliminary evidence on their reliability and validity (*e.g.,* Body Constitution Questionnaire to assess "Stasis" type of BC[79]).

HEALTHY STATUS

World Health Organization (WHO) defines health as "a state of complete physical, mental and social well-being, and not merely the absence of disease or infirmity"; and reproductive health also addresses the reproductive processes, functions and system at all stages of life[80]. This definition is compatible to the concept of TCM in which "Yin and Yang" harmony (balance) makes one "healthy"[79]. "Yin" and "Yang" works together to make body condition or functions in balanced state or status quo (homeostasis), in order to maintain a normal or healthy condition and functioning of the internal organs, emotional state (*e.g.,* free of discomfort), resistance and adaptation to those internal and external stimulations. Figure 1 presents and illustrates the state of the balance between the four main elements of bodily conditions (*i.e.,* "Cool", "Wet", "Dry" and "Heat") in relation to temperature and humidity[81].

Relatively constant body temperature is crucial to maintain homeostasis. Because stable body temperature will allow normal enzyme function[82] and indirectly affect the level of body fluid. The level of humidity and temperature in the human body varies in terms of both the internal and external stimulations, resulting in different combinations of physical, chemical and biological reactions inside the body. "Yang" is the function of the internal organs accounting for producing heat, while "Yin" is the flow of substances or fluids throughout the body or its organs such as water, blood, cells, and endocrine se-

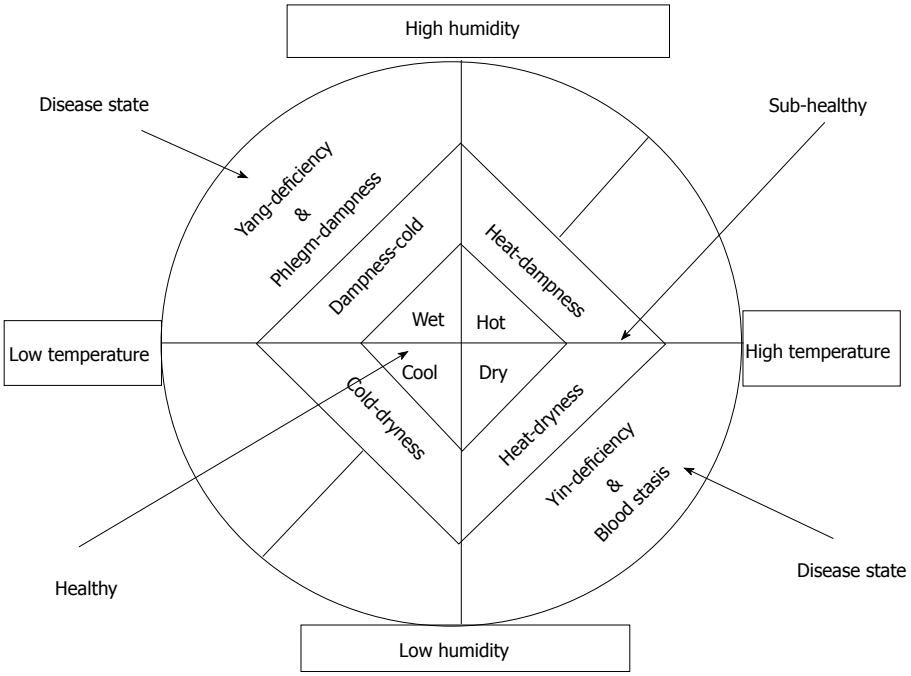

Figure 1 Diagram showing the types of body constitution in relation to health, sub-health and disease status.

cretions in order to support their functioning ("Yang")[83]. Therefore, both "Yin" and "Yang" contribute to maintain stable temperature within the human body[79]. "Yin-Yang" balance refers to the harmony in nature, internal, mental, and physical conditions or statuses, and thus the whole body is in a well-functioned and optimal state to tackle any stimulation against the thresholds of body organs, tissues and other functional units[23]. Such type of "healthy" BC should include the "Heat, Cool, Dry and Wet (dampness)" dimensions in a somewhat balanced state. For example, when the external temperature is high, a few cups of water can replenish the evaporated body fluid, and allay one's thirsty, in line with the concept of "health and illness continuum". Human body can adapt to temperature changes by using a natural physiological response such as sweating and shivering[82] as "Heat adaptation"[84] by a series of reactions in cardiovascular, sudomotor and neuro-endocrine of the body. As long as the "Yin" and "Yang" is in a balanced state, the interrelated bodily mechanisms can work well together to maintain the body condition and functions as "healthy" or normal; otherwise, when beyond the thresholds, the body will at risk of "sub-health", or crossing the border from a "normal" to an "abnormal" BC[24].

Sub-health status

Sub-health, or called the "third state", is an individual's health condition between healthy and being ill as a result of mal-adaptive or abnormal bodily reactions toward internal and/or external stimuli such as infectious diseases, environmental pollution and stressful life situations[85]. This concept is to describe those who are presenting with a few observable subjective complaints such as low and depressed mood, irritability, anxiety, fatigue and muscle pain, but their illness condition cannot be confirmed or diagnosed in Western medicine[86], due to negative results in medical and clinical investigations.

However, the human body at this pre-morbid stage (sub-health) is definitely at risk of some types of diseases due to the person's inability of keeping his bodily condition in homoeothermic or homeostatic state[85,86]. Biochemical and psychological regulatory mechanisms inside our body that can sustain our life and maintain harmony could be lost when the person is unable to maintain a stable core temperature (around 97.6-99.6 ℃ or 36.5-37.5 ℃ and bodily functions such as normal cardiovascular and excretory functions. For instance, when body temperature exceeds the threshold in case of the internal buffering system cannot working properly, the core temperature will be raised; and such elevated core temperature can have heat-associated symptoms such as muscle weakness, lightheadedness, dizziness, sleepiness, fatigue, and confusion[87,88]. Without proper adjustment of body temperature by homeostatic mechanisms such as by sweating and vasodilatation, the person will feel discomfort (when skin temperature beyond the normal range of 36.5-37.5 ℃)[89], and even present increasing levels of disabilities and dysfunctions of various body parts when persistent changes in external or internal temperature occur. According to the theories of TCM, "Cold" (Cool) and "Heat" (Hot) nature of BC in response to changes in body temperature would eventually induce pathological changes inside the human body[90].

Therefore, BC can shift from healthy to unhealthy patterns according to the patterns of symptom differentiation, including "Cold" *vs* "Heat", "Dampness" *vs* "Dryness", "Heat-dampness" *vs* "Cold-dampness" at different levels (low *vs* high) of temperature and/or humid-

ity, which are the important parts of natural environment that the person is situated. Dry and hot environment can induce biochemical reactions inside the body to become "Heat" and "Dryness" patterns of BC; but if the body can adapt to this environment and then cool down the bodily functions, the person will present with "Cool"and "Wet" patterns of BC. Before showing full adaptation to any of these stimuli, respective types and levels of clinical manifestations from mild discomfort to heavy headache and fainting may co-exist according to the pathological changes in the human body (*i.e.,* so called BC in TCM). When a person presents mild discomfort or vivid changes in BC, it is not usually, or sometimes difficult, for a Western medical practitioner to prescribe treatment for the person without any medical diagnosis. However from the TCM perspective, inadequate rest, abnormally high or low external temperature and humidity, and/or extreme internal, emotional fluctuations can cause harmful effects to the body if not early intervened. It is because these can disrupt the balance of "Yin and Yang" and change the individual's BC, thus imposing him/her a higher risk of illness occurrence[90].

In contrast to WHO's definition, people are generally considered healthy when they are in absence of a medical diagnosis, or an obvious disease condition. In a recent study of 4832 Chinese people in Hong Kong, 83% perceived "satisfactory health status" irrespective of 27% having strong risk factors of chronic diseases such as obese, hypertension, hyper-cholesterolaemia and diabetes[91]. Two studies of 400[92] and > 2000[93] so called "healthy" people (without clinical significant signs and symptoms) found that only 38.75% and 35% of them were classified with "healthy" BC, respectively; and the remaining majority were categorized having one of the "abnormal" types of BC, including "Fluid insufficiency", "Internal-heat" and "Heat-dumpness". "Heat-dampness" might also occur when their body temperature was high with fluid overload, resulting in hot feeling, obesity, very tiredness, bitty taste in mouth, and massive yellowish vaginal discharge[94] . In longer term, such type of BC tends to develop obesity[95], and at risk of various chronic diseases, such as diabetes mellitus[96] and coronary heart disease[97]. With increasing research on BC, the concept of "sub-health" seems to be emerging and showing similarities to the pre-clinical or pre-morbid stage of diseases in Western medicine, but receiving much attention by TCM practitioners. Sub-health condition should be assessed and thus the person would be provided with preventive measures for disease occurrence and related suffering in future.

DISEASE STATUS

Further to the changes in BC discussed above for "sub-healthy" state, more pathological changes in BC would develop into "Yang" Deficiency, presenting with subnormal temperature and/or overload of body fluid[98], and subsequently proceeding to disturbance of endocrine secretions such as hormones in the hypothalamic pituitary adrenal and/or thyroid axis[99]. Inability of standing with biological and psychological stressors would result in disease occurrence. On the other hand, "Yin-deficiency" is due to "Heat" and "Dryness", as well as inability of replenishing body fluid after an excess loss[83]. The core temperature would then be increased and eventually resulted in cell and tissue damage, which is found compatible to the theory of tissue and cell inflammation and damage as the result of hyperthermia in Western medicine[100]. In addition, "Blood stasis" due to unsatisfactory systematic circulation can occur if body temperature and humidity are low, presenting with feeling of numbness over the body, poor circulation, cool extremities, and purple lips[83], while "Phlegm-dampness" can be due to low temperature with high humidity in which people will present with cold feeling, edema, abnormal tiredness, and poor digestive function[94]. These types of "unhealthy" BC reveal the overall concept of "Yin and "Yang" imbalance, which absolutely contributes to the sickness or disease occurrence.

Recent studies also reported that cancer patients with "Yin-deficiency" BC, which might present with higher heart rate and inability of regulation of body temperature, would increase morbidity and mortality over a few months of follow-up[101]. "Yin-deficiency" type of BC with inadequate body fluid and high body temperature was evidenced to be one of the risk factors of dementia[68] and hypertension[102]. "Yang-deficiency" BC with low temperature and high fluid level, at risk to have osteoporosis after menopause[70] and cancer[103].

BODY CONSTITUTION AND DISEASE PREVENTION

It is important to understand the concept of BC and to consider this to be applied to the prevention and treatment of chronic illnesses, whereas medical treatment in Western medicine may not be able to early detect, improve, maintain, or cure these illnesses. When the global population continues aging, the incidences and prevalence of chronic diseases will certainly increase. Prevention and early intervention strategies in these illnesses from the perspectives of TCM (and BC) should be carefully considered, particularly for those presenting with only pre-clinical or pre-morbid features and, at the same time, non-significant or negative results in clinical examinations. As emphasized by TCM, "Prevention is always better than cure"[6].

Despite different views of BC found in recent research, BC can reflect the current body condition and reveal the functions of internal organs by direct observation of their signs or subjective complaints with four classical examination skills of TCM[30]. Based on the principles of TCM, once these signs of illness appeared, appropriate early treatments or interventions would be needed for improving the individual's BC. Yet, the abnormal BC would be modified and improved (converted or

changed to a "healthy" BC) with herbal medication[7,8,19], as well as diet, nutrition and lifestyle behaviors[21,104]. Researchers[105] conducted a dietary intervention study to improve an abnormal ("Yin-deficiency") BC in 48 people with hypertension. Their results indicated the changes in dietary practice among the hypertensive subjects could modify their abnormal BC to become a "healthy" BC and at the same time, improved their pathological condition such as a decrease in hypertension and its related drug use.

During "sub-healthy" status, lifestyle and diet modifications may help the body refrain from deterioration of health condition or disease occurrence. Eating well with good bowel habit, sleeping well and doing exercise regularly are considered a few key elements to maintain body resistance against disease, from the TCM perspective[22]. In Western culture, healthy eating is based on the concept of the "food pyramid" such as balanced diet or less fat and oils and more fruit and vegetables. In Chinese culture under the influence of TCM, foods with different combinations of "Cold" and "Hot" properties are thought to maintain harmony and a balance of "Yin and Yang" in our body[106]. Food intake according to an individual's BC may be the best way to enhance good health and longevity and prevention of diseases[107].

All along, the medical terms used for pattern differentiation in TCM have been adopted to set up the categories of "abnormal" or "pathological" BC. As there are more than 60 categories of patterns and many known classifications of BC[10], many of these patterns for differentiations are not consistent, still developing and modifying with TCM research over the last decade. However, it is useful to develop and use a simple, clear and user-friendly classification of BC with strong evidence-based patterns, in order to let the TCM and healthcare professionals and the public better understand and use of the concept of BC in detecting "sub-health" and ill health or disease.

CONCLUSION

This article critically discussed the BC, "sub-health", health, and disease conditions of an individual. Recent evidence in TCM supports that an individual's body condition can be influenced by multiple internal and external factors, mainly including genetic, biochemical, ethnic, family, environmental, psychological and behavioral domains. With better categorized "healthy" and "unhealthy" BC patterns, a strong and weak bodily resistance, an adaptive and mal-adaptive functioning of internal organs, and thus a balance of "Yin" and "Yang" in the human body, can be easily identified and differentiated. Healthy people can easily adjust their body condition below the functional thresholds to prevent illness and stress reactions, and thus be more able to adapt to the changes in their internal and external environments, particularly temperature and humidity. Otherwise, less healthy people will shift from a healthy to a "sub-healthy" condition and then proceed to disease status if not appropriately early intervened. To be complimentary with health promotion and disease prevention in Western medicine, understanding about one's BC in TCM, together with its important determinants (*e.g.*, healthy eating and lifestyle behaviors), can contribute to a more proactive, holistic and individualized care in the community.

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P- Reviewer: Zhang SC **S- Editor:** Zhai HH
L- Editor: A **E- Editor:** Wu HL

World Journal of Translational Medicine

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.5528/wjtm.v2.i3.67

World J Transl Med 2013 December 12; 2(3): 67-74
ISSN 2220-6132 (online)

ORIGINAL ARTICLE

Molecular recognition of live *methicillin-resistant staphylococcus aureus* cells using DNA aptamers

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Received: August 09, 2013 Revised: November 11, 2013
Accepted: November 20, 2013
Published online: December 12, 2013

Abstract

AIM: To generate DNA-aptamers binding to *Methicillin-resistant Staphylococcus aureus* (*MRSA*).

METHODS: The Cell-Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technology was used to run the selection against *MRSA* bacteria and develop target-specific aptamers. *MRSA* bacteria were targeted while *Enterococcus faecalis* bacteria were used for counter selection during that process. Binding assays to determine the right aptamer candidates as well as binding assays on clinical samples were performed through flow cytometry and analyzed using the FlowJo software. The characterization of the aptamers was done by determination of their K_d values and determined by analysis of flow data at different aptamer concentration using SigmaPlot. Finally, the recognition of the complex Gold-nanoparticle-aptamer to the bacteria cells was observed using transmission electron microscopy (TEM).

RESULTS: During the cell-SELEX selection process, 17 rounds were necessary to generate enrichment of the pool. While the selection was run using fixed cells, it was shown that the binding of the pools with live cells was giving similar results. After sequencing and analysis of the two last pools, four sequences were identified to be aptamer candidates. The characterization of those aptamers showed that based on their K_d values, DTMRSA4 presented the best binding with a K_d value of 94.61 ± 18.82 nmol/L. A total of ten clinical samples of *MRSA*, *S. aureus* and *Enterococcus faecalis* were obtained to test those aptamers and determine their binding on a panel of samples. DTMRSA1 and DTMRSA3 showed the best results regarding their specificity to *MRSA*, DTMRSA1 being the most specific of all. Finally, those aptamers were coupled with gold-nanoparticle and their binding to *MRSA* cells was visualized through TEM showing that adduction of nanoparticles on the aptamers did not change their binding property.

CONCLUSION: A total of four aptamers that bind to *MRSA* were obtained with K_d values ranking from 94 to 200 nmol/L.

Key words: Aptamer; *Methicillin-resistant Staphylococcus aureus*; Gram-positve bacteria; Cell recognition

Core tip: *Methicillin-resistant Staphylococcus aureus* (*MRSA*) is a nosocomial bacterium that has developed resistance to beta-lactam antibiotics and can now be contracted in community settings. A tool that would enable the recognition of *MRSA* through its membrane structure could lead to new therapeutic approaches to eradicate the *MRSA* superbug. This paper presents four

MRSA aptamers that can be easily modified as molecular probes for bioanalysis or antibiotics-free therapy. The Cell-SELEX technology was used to develop target-specific aptamers and binding studies of those aptamers were performed by flow cytometry on a panel of clinical strains. A total of four aptamers that bind to *MRSA* were obtained.

Turek D, Van Simaeys D, Johnson J, Ocsoy I, Tan W. Molecular recognition of live *methicillin-resistant staphylococcus aureus* cells using DNA aptamers. *World J Transl Med* 2013; 2(3): 67-74 Available from: URL: http://www.wjgnet.com/2220-6132/full/v2/i3/67.htm DOI: http://dx.doi.org/10.5528/wjtm.v2.i3.67

INTRODUCTION

The resistance of bacteria to antibiotics is a major public health concern. In particular, *Staphylococcus aureus (SA)* is a bacterium harmlessly carried in the nose or on the skin of about 33% of the population[1]. Nevertheless, this nosocomial-acquired pathogen sometimes causes an infection. This microbe's primary mode of transmission is by direct contact, usually skin-to-skin, and it can cause skin and wound infections, as well as life-threatening infections, including pneumonia, bacteremia or endocarditis[2]. *SA* infections are usually treated using β-lactam antibiotics, *e.g.*, methicillin and penicillin, which inhibit the construction of the cell wall of Gram-positive bacteria, such as *SA*[3]. Unfortunately, *SA* easily develops antibiotic-resistant strains, and is, therefore, often referred to as a "superbug". With the discovery of penicillin by Alexander Fleming in 1928, a dramatic reduction in mortality rates for *SA* infections occurred until resistant strains began to appear not long thereafter[4]. Many more antibiotics have been developed since then, but antibiotic-resistant *SA* strains continue to emerge. Aside from resistance, the use of antibiotics, in general, raises safety concerns since long-term therapy can result in damage to patients' commensal flora[5-7]. The impact on human health is becoming very important since *Methicillin-resistant Staphylococcus aureus* (*MRSA*), which used to be confined to hospital settings, is now prevalent in community settings[8,9]. *MRSA* is currently detected using multiplexed PCR primers that detect specific genes for *S. aureus* (*e.g.*, *nuc* or *fem*) and *mecA* for detection of methicillin resistance[10]. The eradication of multidrug-resistant bacteria is very difficult; thus, it is essential to look for treatment options other than antibiotics. To address this challenge, we used a technology known as Systematic Evolution of Ligands by Exponential Enrichment (SELEX) to generate four *MRSA* strain-specific aptamers that can easily be modified as an antibiotics-free therapeutic modality. This cell-based selection strategy generates ssDNA aptamers that bind to unknown targets on the surface of the cell membrane.

MATERIALS AND METHODS

SELEX library and primers

The library consisted of a 40 bases randomized region flanked by primer regions, each consisting of 18 nucleotides (5'-ATC CAG AGT GAC GCA GCA $(N)_{40}$ TGG ACA CGG TGG CTT AGT-3'). The forward primers were labeled with 5'-FITC, and the reverse primers were labeled with 5'-biotin. Fluorescein isothiocyanate (FITC) labeling enabled fluorescence monitoring during selection, while the biotin was used for separation of the sense strand from the antisense strand after polymerase chain reaction (PCR) amplification through streptavidin-biotin interaction and subsequent alkaline denaturation. All oligonucleotides were synthesized by standard phosphoramidite chemistry using an ABI 3400 DNA synthesizer (Applied Biosystems) and purified by reverse phase High-performance liquid chromatography (HPLC) (Varian Prostar).

PCR and flow cytometry instrumentation and experimental conditions

All PCR mixtures contained 50 mmol/L KCl, 10 mmol/L Tris HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, deoxyribonucleoside triphosphates (each at 2.5 mmol/L), 0.5 μmol/L each primer, and Hot start Taq DNA polymerase (5 units/μL) (TaKaRa). Amplification was carried out on a BIO-RAD thermocycler at 95 ℃ for 30 s, 60.7 ℃ for 30 s, and 72 ℃ for 30 s, followed by the final extension for 3 min at 72 ℃. Pool enrichment was monitored by flow cytometry analysis using a FACScan cytometer (BD Immunocytometry Systems). Dulbecco's Phosphate Buffered Saline (PBS) was used to prepare the washing buffer (4.5 g/L glucose and 5 mL $MgCl_2$ at 1 mol/L) and binding buffer (4.5 g/L glucose, 5 mL $MgCl_2$, 1.0 g/L bovine serum albumin and 100 mg/L tRNA).

Bacteria strains and bacteria culture

MRSA standard strain 43300, was purchased from ATCC, and a clinical strain of *Enterococcus faecalis* was obtained from the Emergency Pathogen Institute at the University of Florida. *MRSA* was cultured at 37 ℃ in ATCC medium 18 - Trypticase soy agar with addition of 4 mg/L of sterile methicillin in order to maintain the resistance structural property of the bacteria. *Enterococcus faecalis* was cultured at 37 ℃ in ATCC medium 260 - Trypticase soy agar with defibrinated sheep blood and in the corresponding broth with no blood. Stock solutions of each cell line were prepared for the selection study and optimized throughout four solutions. The best results were obtained by the following procedure. Cell lines were incubated overnight at 37 ℃ on their respective agar plate. Then 3-4 bacterial colonies were transferred from the agar plate to 15 mL Corning centrifuge tubes filled with 6-7 mL of corresponding broth and incubated overnight at 37 ℃. From this stock, 3 drops of bacterial solution were transferred to another 15 mL Corning centrifuge tube containing 4 mL of corresponding broth and incubated for 3 h. These two batches of incubated cells

were separately washed twice with PBS and fixed in 70:30 methanol: DNase-free water. As a precaution to minimize the clumping, DNase-free water was added first, and cells were resuspended, followed by the corresponding volume of methanol. After 2 h of fixation at 4 ℃, cells were stored in 10% PBS: DNase-free water. Finally, OD600 was measured for both batches, and a mixture was made to get the lowest OD600 measured out of the two batches. Four stock solutions of each bacterium were used for the entire selection. For *MRSA*, stock solution 1: OD600 = 2.08, 15 mL; stock solution 2: OD600 = 1.03, 22 mL; stock solution 3: OD600 = 1.73, 18.7 mL; stock solution 4: OD600 = 1.58, 59.5 mL. For *Enterococcus faecalis*, stock solution 1: OD600 = 1.18, 12.5 mL; stock solution 2: OD600 = 0.762, 17.5 mL; stock solution 3: OD600 = 2.49, 14 mL; stock solution 4: OD600 = 2.27, 66 mL.

In vitro cell-SELEX procedure

In this selection, Methicillin-resistant *MRSA* standard strain 43300 was used as the target with *Enterococcus faecalis* as a negative control. Besides the first round where 10^7 cells were incubated with 22 nmol of naive ssDNA library dissolved in binding buffer, all other rounds were incubated with 25 pmol of the library obtained from the previous round. Before incubation, the DNA library was denatured at 95 ℃ for 5 min and quickly cooled on ice for 10 min, allowing each sequence to form the most stable secondary structure. Each round was performed with the counter selection first with an incubation of 30 min in an orbital shaker at 4 ℃. The supernatant containing the unbound DNA sequence was then incubated with the positive cell line for 1 h in the same conditions. The pellet obtained was then washed two or three times, depending on the round - the stringency of the washes being increased up to 3 washes with 1 mL of washing buffer for 3 min. After suspension of the last pellet in DNase-free water, the pool was denatured at 95 ℃ for 15 min and centrifuged at 14000 *g* for 2 min. The supernatant containing the ssDNA was recovered and amplified by PCR using FITC- and biotin-labeled primers to increase the number of copies of individual sequences. A preparative PCR was performed using the amplified pool as the template. Amplifications were carried out at 95 ℃ for 30 s, 56 ℃ for 30 s, and 72 ℃ for 30 s, followed by final extension for 3 min at 72 ℃. The selected sense ssDNA strands were separated from the biotinylated antisense ssDNA by streptavidin-coated Sepharose beads (GE Healthcare Bioscience). The ssDNA was eluted from the beads by melting in a 0.2 mol/L NaOH solution. This was desalted using NAP5 columns, dried and resuspended in binding buffer to a concentration of 250 nmol/L. The selection process was repeated until the level of enrichment, as assayed by flow cytometry, reached a plateau. Once the plateau was reached, pools of interest were submitted for sequencing using the Ion Torrent technique. The alignment was processed using MAFFT software.

Binding affinity assays

Binding assays were used for two purposes: to assess the potential aptamer candidates and determine their apparent dissociation constant and to screen the aptamers against different cell lines. Binding assays to assess the potential aptamers and screen them against different cell lines used the same protocol. That protocol is based on the target cells, here *MRSA* (10^7 cells), which were incubated with various concentrations of 5'-FITC-labeled aptamers at 4 ℃ for 20 min in 100 μL of binding buffer. The fluorescence intensity was determined by FACScan cytometry (BD Immunocytometry Systems) by counting 60000 events for binding assays and 30000 for K_d determination. Cells only and random library (Library 0) were used as the background signal. The specific binding was obtained by subtracting the mean fluorescence intensity of the random library from the mean fluorescence intensity of the aptamers. The equilibrium dissociation constant (K_d) was obtained by fitting a plot of the specific binding intensity (Y) *vs* the aptamer concentration (X) to the equation $Y = B_{max}X/(K_d + X)$, using SigmaPlot (Jandel, San Rafael, CA). Cell specificity of the selected aptamers was determined using binding assays by flow cytometry monitoring, using *MRSA*, *SA* and *Enterococcus faecalis* clinical cell lines.

TEM visualization of MRSA aptamer binding using gold nanoparticles

The four 5' thiol-labeled aptamers were synthesized for these experiments, as explained in the instrumentation section, and fixed *MRSA* cells were used to run the binding assay. 40 μmol/L initial concentration of the 5' thiol-modified aptamers were conjugated to 1 mL of gold nanoparticles (AuNPs) by incubation for 12 h in 50 μL of 10 mmol/L HEPES buffer (pH 7.5). Then, 100 μL of *MRSA* were mixed with 100 μL of aptamer-AuNPs conjugates and incubated for 1 h at room temperature. After removal of the unbound sequences with water by centrifugation at 3000 *g*, analysis was done by transmission electron microscopy (TEM) using 1% uranyl acetate staining. Assays were run with incubation of cells only, AuNPs only, sgc8-AuNPs only (sgc8 is used here as a random aptamer) and DTMRSA-AuNPs conjugates. Note that the centrifugation speed applied to obtain these results was low (3000 *g*), compared to the usual speed applied when using Au-NPs (10000 *g*). This makes it easier to observe the NPs upon target binding since they can be collected with the cell pellet, whereas nonbinding NPs observed in the control tend to remain in the discarded supernatant.

RESULTS

In-vitro cell-SELEX

In this study, we selected aptamers binding to *MRSA*. Using the SELEX technique, a cell-selection was carried out using a random library of ssDNA that was subjected to sequential binding with the object of selecting those aptamers from the pool of DNA sequences having high binding affinity to surface markers on the target *SA* cell. Counter-selection using the Gram-positive commensal bacterium *Enterococcus faecalis* was performed on each round, allowing us to eliminate common surface markers,

Table 1 Quantitative representation of the different homologous families and *Methicillin-resistant Staphylococcus aureus* aptamer sequences after Ion Torrent sequencing and alignment

Name of aptamers	Percentage of total sequences	Sequence
DTMRSA1	2.57%	ATCCAGACGTGACGCAGC(N)$_{38}$TGGACACGGTGGCTTAGTA (N)$_{38}$ = ATGCGGTTGGTTGCGGTTGGGCATGATGTATTTCTGTG
DTMRSA2	33.74%	ATCCAGAGTGACGCAGCA(N)$_{36}$TGGACACGGTGGCTTA (N)$_{36}$ = CGACACGTTAGGTTGGTTAGGTTGGTTAGTTTCTTG
DTMRSA3	10.05%	ATCCAGAGTGACGCAGCA(N)$_{40}$TGGACACGGTGGCTTAGTA (N)$_{40}$ = GTAGATGGTTTGGTTGGTGTGGTTTCCTACTGATGTTGGG
DTMRSA4	0.32%	ATCCAGAGTGACGCAGCA(N)$_{39}$TGTGGACACGGTGGCTTA (N)$_{39}$ = TTATGGGGTGTGGTGGGGGGTTAATGCGTTGGTTATCCG

The primers used for the selection are presented in bold. Note that some primer sequences changed through the selection process.

Table 2 Relative binding of the selected aptamers to various clinical cell lines

Clinical strains	DTMRSA1	DTMRSA2	DTMRSA3	DTMRSA4
MRSA 2	+++	++++	+++	++++
MRSA 4	+++	+++	+++	++++
MRSA 6	++++	++++	+++	++++
MRSA 7	-	++++	+++	++++
S. aureus 164	-	++++	+	+++
S. aureus 165	-	++++	+	+++
S. aureus 166	-	++++	+	+++
E. faecalis 43	-	++++	+	+++
E. faecalis 44	-	++++	+	++
E. faecalis 45	-	++++	+	+++

This table summarizes the results obtained with the binding assays on clinical strains of *methicillin-resistant Staphylococcus aureus* (*MRSA*), *S. aureus* and *E. faecalis* bacteria. (-) no binding; (+) 0%-25%; (++) 25-50%; (+++) 50%-75%; (++++) 75%-100%. S. *aureus: Staphylococcus aureus; E. faecalis: Enterococcus* faecalis.

while, at the same time, enriching specific markers on the target bacteria. Both negative and positive cell lines were fixed with methanol before being used in the selection process. Four stock solutions of each fixed bacterium were prepared, as explained in the Experimental Section. The first stock solution was used from round 1 to 11; the second from round 12 to 14; and the third from round 15 to 17. The eluted pool of each round was amplified by PCR and monitored by flow cytometry. Since the pools were enriched throughout different rounds with binding aptamers toward *MRSA*, an increase in fluorescent signal was observed. The flow cytometry analysis of enrichment of the libraries and the binding assays with individual aptamers were performed on batch four. By the end of the 14th round of selection, a significant increase of specific pool enrichment was observed. After 15 rounds, a plateau was reached, and the selection was run until round 17 to maximize enrichment and homology within binding sequence families.

Generation of aptamers

After successful enrichment, selection pools 15 and 17 were chosen and prepared for sequencing. Pool 15 represented the point at which enrichment started a plateau observable by flow cytometry, and pool 17 was the end of the plateau. Sequencing was done with IonTorrent, which identified 600000 sequences. In analyzing the data, the 40 nt random regions were aligned using the MAFTT alignment program. The alignment generated several different homologous families, and representative sequences were identified using the MAFTT and mfold oligo analyzer software programs. Putative DNA aptamers were then synthesized, labeled with FAM (carboxyfluorescein), purified by HPLC and quantified. Binding assays were performed for each sequence on *MRSA* cells and *Enterococcus faecalis* cells to determine the relevant aptamer sequences. Initially, we chose all candidates showing observable binding to the target cell *MRSA* when compared with the control - the random library. Positive sequences were further screened with *Enterococcus faecalis* to select the aptamers showing specific binding to *MRSA*. All flow cytometry data were run with unlabeled cells and random library (Library 0) as negative control. As shown in Figure 1 and Table 1, four of those sequences showed specific binding to *MRSA*.

Binding assays with a panel of clinical bacteria strains

Ten clinical strains from Shands Hospital (Florida, United States) were tested against the four aptamers developed (Table 2). DTMRSA1 and DTMRSA3 showed the best specificity and appeared to be the best candidates for *MRSA* treatment investigation, DTMRSA1 being the best one of the two, while DTMRSA2 and DTMRSA4 bound to all three types of clinical bacteria strains.

DISCUSSION

Over the course of the past two decades, mammalian cells have been the focus of most aptamer studies[11-13]. As presented in the result section, we generated four aptamers binding to the *MRSA* bacterium using the SELEX method. The selection was carried out using fixed cells for safety purposes and further investigation was performed to check whether the enriched pools generated from fixed cells could also bind to live *MRSA* cells. In order to verify whether methanol had an adverse effect on the binding of the enriched pools, we compared the binding of fixed cells with that of live cells. As can be observed in Figure 2, the binding was maintained when

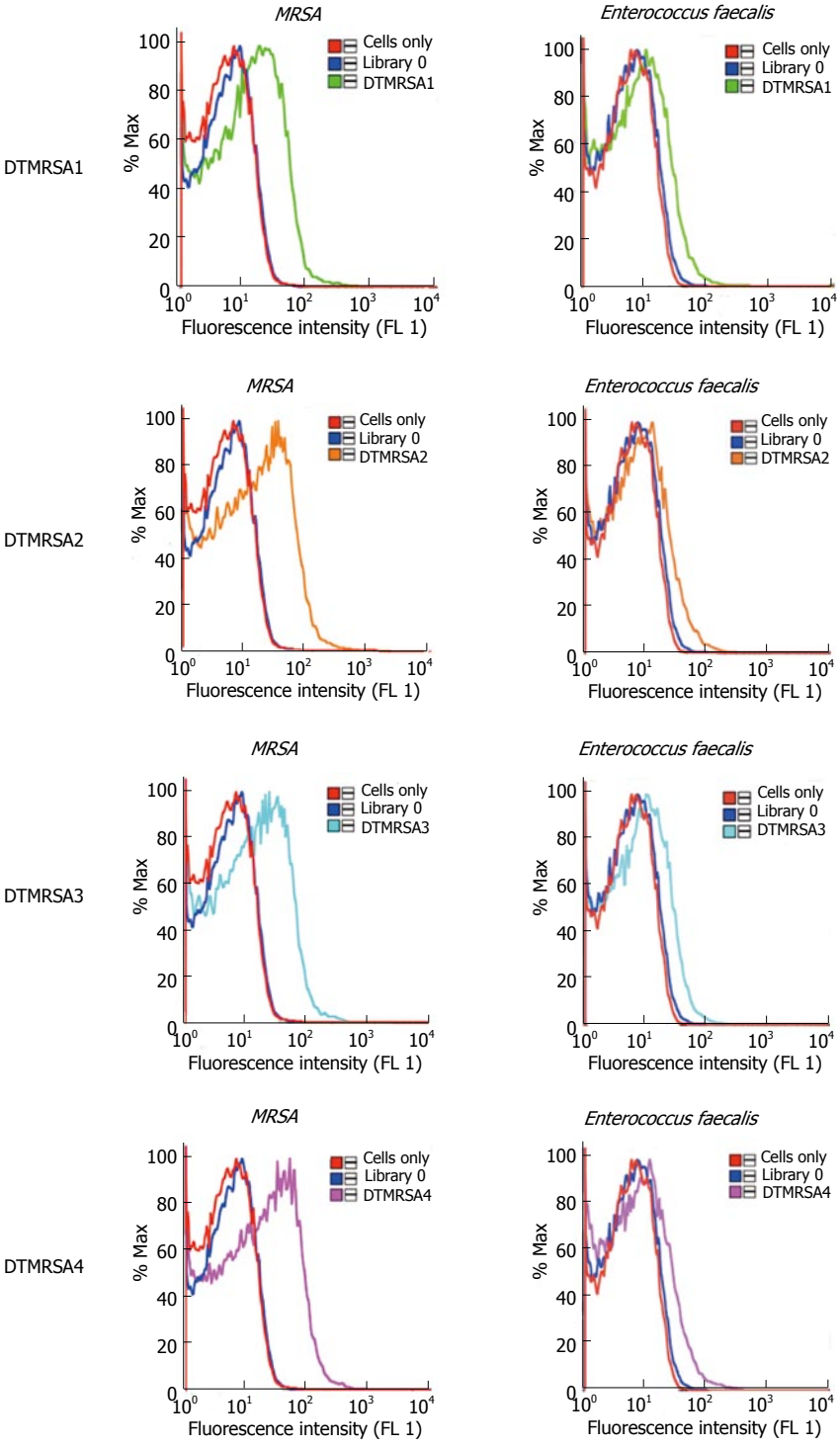

Figure 1 Flow cytometry histograms of the relevant aptamer candidates. Four aptamers show significant binding to *methicillin-resistant Staphylococcus aureus* (*MRSA*) 43300 bacteria, but not to the *Enterococcus faecalis* cell line.

using live cells, demonstrating little permanent adverse effect in the structure of the membrane proteins of the bacteria and the possibility to use fixed cell when running a selection with no counter effect on the binding.

Since the binding was conserved whether live or fixed were used, the pools were sent to sequencing. Through the sequencing analysis, one tends to select for the more abundant sequences. Even though it is the most obvious strategy, the aptamer structure itself is as meaningful for its binding properties as it can be observed with DTMRSA4 that represent only 0.32% of the total sequences but shows a binding as important as DTMRSA2, the most abundant aptamer sequence as shown in Table 1.

Apparent dissociation constants (Kd) are shown in Table 3 and Figure 3: DTMRSA3 and DTMRSA4 show very good binding with apparent dissociation constant (*Kd*) of $1.3 \pm 0.5 \times 10^2$ nmol/L and $9.5 \pm 2 \times 10^1$ nmol/L, respectively.

Apparent dissociation constants have been studied in the past through cell-SELEX performed on bacteria[14,15], and values typically ranged between 30 and 250 nmol/L, similar to those discovered here. We believe that these affinities are sufficient for *MRSA* detection assays. The other important and interesting property of these aptam-

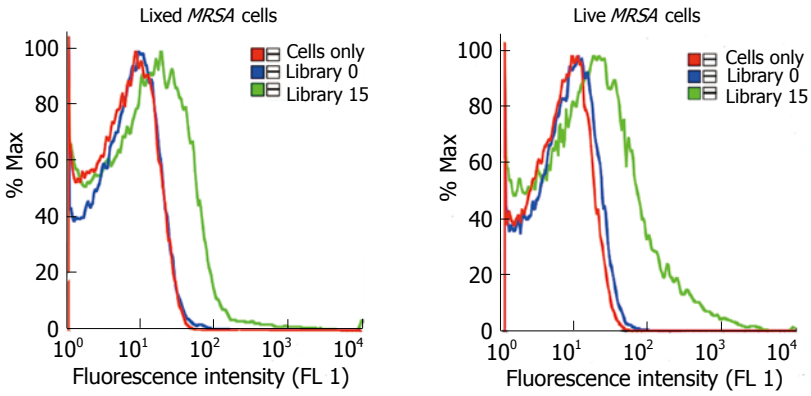

Figure 2 Comparison of the binding results between fixed and live *methicillin-resistant Staphylococcus aureus* cells. Binding assays show that results remain the same, irrespective of whether experiments are run with fixed or live cells. MRSA:*Methicillin-resistant Staphylococcus aureus*.

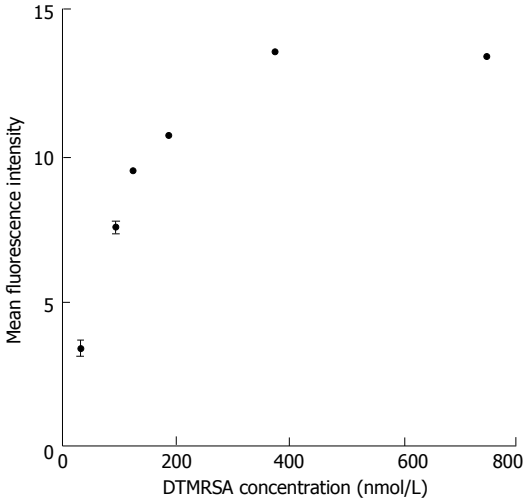

Figure 3 SigmaPlot binding curve of the aptamer DTMRSA4 used for the *Kd* value determination. *Methicillin-resistant Staphylococcus aureus* cells were incubated with various concentrations of fluorescein isothiocyanate-labeled DTMRSA4.

Table 3 Apparent dissociation constants (*Kd*) of the four aptamers recognizing *methicillin-resistant Staphylococcus aureus* bacteria

Name of the aptamer	*Kd* (nmol/L)
DTMRSA1	$1.6 \pm 0.5 \times 10^2$
DTMRSA2	$2.0 \pm 0.6 \times 10^2$
DTMRSA3	$1.3 \pm 0.5 \times 10^2$
DTMRSA4	$9.5 \pm 2 \times 10^1$

ers is their ability to selectively bind around whole cells, which presents a potential clinical use of the selected aptamers as nanocarriers for the identification of antibiotic-resistant cell lines in patients. The successful development of an assay that can differentiate the resistance of *SA* colonies can facilitate the search for a more effective treatment and management of the disease in individual treatment or in the population.

The latest estimations of the occurrence *MRSA* are staggering, rising to epidemic proportions in hospitals where, by an estimate provided by the European Antimicrobial Resistance Surveillance System, 40%-60% of all infections were caused by *S. aureus* in the United States and United Kingdom[16,17]. The ability of the *MRSA* membrane to structurally change through time in order to survive is a characteristic that increases its lethality and decreases its controllability[18,19]. Consequently, the binding ability of these four aptamers was compared in the context of different clinical *MRSA* strains, as well as *SA* and *Enterococcus faecalis* (Table 2). We are encouraged that the selected aptamers bound to all *MRSA* clinical strains tested, suggesting that the proteins targeted by those aptmers are common for the different *MRSA* strains. *MRSA*, *SA* and *Enterococcus aureus* are all gram-positive bacteria. Based on their similarities, some common binding can be expected by the commonality of proteins among the strains, whereas others are more specific to each type of bacterium. DTMRSA1 and DTMRSA3 show the best specificity and therefore appear to be the best candidates for *MRSA* treatment investigation, DTMRSA1 being the best one of the two, while DTMRSA2 and DTMRSA4 bind to all three types of bacteria.

Therefore, DTMRSA1-4 collectively represent a powerful tool by which to study the membrane structure of *MRSA*, as well as develop potential treatment modalities to combat this pathogen. As an empirical example of such use, we ran a prelimanary study to visualize the binding sites of *MRSA* aptamers on the bacteria external membrane. To accomplish this, we conjugated *MRSA* aptamers to AuNPs, and we were able to observe their target binding *via* TEM. Flow cytometry is one of the best tools to measure the abundance of a membrane protein on cells[20]. The introduction of a nanoparticle-aptamer conjugate detectable by TEM instead of a dye, as used in flow cytometry, could be a valuable adjunct to complement the information we get from the expression of aptamer targets on the bacteria. This technique allows the visualization of both the binding site of aptamers upon target binding and the structure of the cell wall[21-23]. No binding between *MRSA* and bare gold or random aptamer conjugated to gold nanoparticles was observed, whereas DTMRSA2-AuNP conjugates were attached to the surface of *MRSA*, as detected by TEM. With this successful visualization of the aptamers on *MRSA* cells, we showed that the adduction of nanoparticles on the aptamers did not change their binding property. Since nanoparticles have unique properties of their own[24,25], we believe this approach can be push further and lies on the choice of nanoparticles to either study the morphol-

ogy of the bacteria, detect it or eradicate it.

In conclusion, once confined to hospital settings, *MRSA* can now be contracted in community settings as well. Although many new antibiotics against *MRSA* are in phase Ⅱ and Ⅲ clinical trials, a tool that would enable the recognition of *MRSA* through its membrane structure could lead to new therapeutic approaches to eradicate the *MRSA* superbug, either without the use of antibiotics or with a strain-specific antibiotic. The possibility of using the SELEX technique on fixed bacteria cells to develop aptamers binding to *MRSA* that will show the same results on live bacteria cells was shown in this paper. Four aptamers were found to recognize the bacteria membrane, DTMRSA1 and DTMRSA3 being the most specific when a wide range of clinical bacteria strains were tested, and DTMRSA4 presented the strongest binding to *MRSA* cells based on its K_d value. The binding of those aptamers were confirmed after modification with nanoparticles and visually observed through transmission electron microscopy showing those aptamers could be ealily modified to serve as molecular probes for bioanlysis or antibiotics-free therapy. Further studies would expand the present work to an optimized shorter aptamer length and a better understanding of the aptamer target on the membrane of *MRSA* bacteria.

ACKNOWLEDGMENTS

We thank Mohammed Rashid for the bacteria training and scientific discussions that contributed to this work. We also thank David Nolan and Dr. Marco Salemi for providing clinical cell lines. Finally, we thank Dr. Kwame Sefah and Dr Kathryn R. Williams for their critical review of the manuscript.

COMMENTS

Background

Methicillin-resistant Staphylococcus aureus (*MRSA*) is any strain of *Staphylococcus aureus* that has developed resistance to beta-lactam antibiotics, including the penicillins and the cephalosporins. Once confined to hospital settings, *MRSA* can now be contracted in community settings as well.

Research frontiers

Many new antibiotics against *MRSA* are in phase Ⅱ and Ⅲ clinical trials, nevertheless, a tool that would enable the recognition of *MRSA* through its membrane structure could lead to new therapeutic approaches to eradicate the *MRSA* superbug, either without the use of antibiotics or with a strain-specific antibiotic.

Innovations and breakthroughs

In the recent years, a cell selection has been done on Staphylococcus aureus and a protein selection has been done on Enterotoxin B, but has not been tested on whole cells. Antibodies are available against *MRSA* but do not present as much flexibility and advantages as aptamers.

Applications

The development of *MRSA* aptamer would present a great tool to new therapeutic approaches to eradicate the *MRSA* superbug, either without the use of antibiotics or with a strain-specific antibiotic.

Peer review

This article is considered to be useful for others research scientists working in the fields of aptamers or *MRSA* bacteria.

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P- Reviewers: Grizzi F, Pranjal C **S- Editor**: Song XX
L- Editor: A **E- Editor**: Wu HL

World Journal of Translational Medicine

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.5528/wjtm.v2.i3.75

World J Transl Med 2013 December 12; 2(3): 75-82
ISSN 2220-6132 (online)

BRIEF ARTICLE

"Diabegon", a safe and effective polyherbal therapy for type 2 diabetes mellitus

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Supported by AYUSH, New Delhi, India, No. F.NO. Z.31014/02/2009/EMR-CCARS; and M/S Deendayal Aushadhi Pvt. Ltd. Gwalior, India

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Received: August 25, 2013 Revised: October 21, 2013
Accepted: November 20, 2013
Published online: December 12, 2013

Abstract

AIM: To investigate the antihyperglycemic, antihyperlipidemic and antioxidant functions of a polyherbal formulation, "Diabegon", in human subjects with type 2 diabetes mellitus.

METHODS: A total of 33 human subjects with type 2 diabetes mellitus were recruited for the study and all anthropological and biochemical parameters were recorded at the time of registration. The subjects were given hot water extract obtained from 10 gm of "Diabegon" powder, "Diabegon kwath", on an empty stomach everyday in the morning under personal supervision for 6 mo. The therapeutic functions of the "Diabegon kwath" was assessed by monitoring the blood glucose levels at monthly intervals and glycosylated hemoglobin, lipid profile and biomarkers of oxidative stress, liver and kidney function markers at three monthly intervals in the study subjects.

RESULTS: Daily administration of hot water extract of "Diabegon" regularly for 6 mo resulted in significant reductions of blood glucose and glycosylated hemoglobin levels. There was also a significant increase in high density lipoprotein cholesterol levels with concomitant decreases in total cholesterol, triglycerides, low density lipoprotein cholesterol and very low density lipoprotein. A significant improvement in glycosuria and proteinuria was also observed. Also, the subjects exhibited a significant improvement in enzymatic and nonenzymatic biochemical markers of oxidative stress. The kidney and liver functions remained normal and in fact improved in many subjects.

CONCLUSION: The study which is first of its kind, advocates "Diabegon kwath" as a safe and effective Ayurvedic therapy for the treatment of human type 2 diabetes mellitus and further placebo controlled trial may substantiate the therapeutic efficacy of the formulation.

Key words: Type 2 diabetes mellitus; Diabegon kwath; Polyherbal formulation; Oxidative stress; Blood glucose; Lipids; Antiglycemic; Antihyperlipidemic; Antioxidant; antidiabetic therapies

Core tip: The study evaluated antiglycemic, antihyperlipidemic and antioxidant functions of a polyherbal formulation designated "Diabegon kwath" in type 2 diabetic subjects with varying degrees of hyperglycemia and found that the formulation serves as an effective alternative to conventional antidiabetic therapies.

Mahajan S, Singh N, Subramanian SK, Chauhan P, Saxena S, Goswamy HM, Prasad GBKS, Bisen PS. "Diabegon", a safe and effective polyherbal therapy for type 2 diabetes mellitus. *World J Transl Med* 2013; 2(3): 75-82 Available from: URL: http://www.wjgnet.com/2220-6132/full/v2/i3/75.htm DOI: http://dx.doi.org/10.5528/wjtm.v2.i3.75

INTRODUCTION

Type 2 diabetes mellitus is one of the most common global metabolic disorders and is characterized by abnormalities of carbohydrate and lipid metabolisms, mainly resulting either from defects in insulin secretion and/or insulin action, or adipocyte functioning[1]. Although the disease manifests in the form of hyperglycemia, the cause can vary, ranging from disturbance in insulin secretion, insulin action, insulin resistance, glucose production and glucose uptake, interplay among different metabolic pathways, hormones, *etc.* Type Ⅱ diabetic patients often exhibit increased low density lipoprotein (LDL) and decreased high density lipoprotein (HDL) cholesterol levels and hypertension, as well as altered platelet function[2]. Due to such varied etiology, not a single agent or molecule has so far been unequivocally accepted as the antidiabetic drug. There is a broad range of glucose and lipid-lowering (metformin, sulfonylureas, insulin, statins) drugs which although are successful to some extent, careful consideration must be given when selecting the appropriate glucose and lipid-lowering therapy. The conventional antidiabetic therapies are reported to be associated with many side effects, such as hypoglycemia, lactic acid intoxication and gastrointestinal upset[3], in diabetic subjects. Statins are very widely used during dyslipidemia and there are reports in experimental models that statin therapy may exhibit an adverse effect on glucose homeostasis[4].

Indigenous systems of medicine based on traditional wisdom have thrived through the ages and are practiced by a large population all over the globe for the management of diabetes. A large number of plants have proved their efficacy in the management of hyperglycemia, hyperlipidemia, oxidative stress and the inflammatory response[5-7]. Scientific validation of many of the plant-based antidiabetic medicines has been done[8-10] and the bioactive principles identified and characterized[11,12]. The antihyperglycemic effect of several plant extracts and herbal formulations that are used as antidiabetic remedies has been confirmed[13,14]. Plant drugs are frequently considered to be less toxic and free from side effects than synthetic ones[15]. Combined extracts of herbs are used as the drug of choice rather than individual plant extracts. Herbal formulations[16] were shown to exhibit antidiabetic, antioxidant effects in animal models as well as in diabetic subjects[17,18]. The phytochemical based formulations consist of multiple herbs and are therefore liable to produce a large number of metabolites that may act on multiple targets in the body. Although the phytochemical formulations have been widely used for many years, systematic scientific evidence and proof of efficacy are generally lacking compared with synthesized chemical medicines. Diabegon powder, a plant based formulation consisting of a mixture of about 10 herbs, *Gymnema sylvestre* (*Gurmar*), *Eugenia jambolana* (*Jamun seed*), *Emblica officianale* (*Amla*), *Curcuma longa* (*Haldi*), *Pterocarpus marsupium* (*Vijaysaar*), *Terminalia chebula* (*Harad*), *Cassia fistula* (*Amaltas*), *Picrorhiza kurroa* (*Kutki*), *Swertia charita* (*Chiraita*) and *Terminalia Bellerica* (*Behada*), was validated in this study for its therapeutic potential in human type Ⅱ diabetes mellitus.

MATERIALS AND METHODS

Subjects

A total of 33 type Ⅱ diabetic subjects attending a weekend diabetes clinic run by the School of Studies in Biochemistry, Jiwaji University, India were randomly selected for the study after giving informed written consent. The following criteria were employed for selecting the subjects for the study.

Inclusion criteria

1. Non-insulin dependent diabetics diagnosed as per the criteria of World Health Organization;
2. Both genders between the ages of 30-65 years;
3. Body Mass Index range between 18.5 and 30;
4. Participants who understood the benefits of the study and signed a written informed consent.

Exclusion criteria

1. Presently using other blood glucose level controlling agents;
2. Daily intake of alcoholic beverages;
3. Smokers consuming more than 1 pack/d;
4. Patients diagnosed as type Ⅰ and type Ⅱ diabetes mellitus (insulin requiring stage);
5. Patients with ketosis, diabetes related complications, hepatic or renal disease, pancreatitis, cardiac problems, uncontrolled hypertension, malnutrition and severe immune deficiency.

The subjects had the objectives, nature of drugs, rationale and duration of therapy to be administered explained to them in the local language. They were asked to avoid a carbohydrate rich diet and regular walking for about 4-5 km during the course of therapy was advocated. Anthropometric measurements like weight, height and waist were recorded at monthly intervals. The patients were kept exclusively on "Diabegon therapy" and did not take any other kind of oral antihyperglycemic or lipid lowering drugs during the study period.

Drug, doses and duration

The drug administered is purely a polyherbal formulation consisting of *Gymnema sylvestre* (*Gurmar*), *Eugenia jambolana* (*Jamun seed*), *Emblica officianale* (*Amla*), *Curcuma longa* (*Haldi*), *Pterocarpus marsupium* (*Vijaysaar*), *Terminalia chebula* (*Harad*), *Cassia fistula* (*Amaltas*), *Picrorhiza kurroa* (*Kutki*), *Swertia charita* (*Chiraita*) and *Terminalia bellerica* (*Behada*)

Table 1 Effect of 6 mo polyherbal therapy on hyperglycemia

Biochemical parameter	Variations at 3 monthly intervals following polyherbal therapy			
	0 d	3 mo	6 mo	Mean change
Fasting plasma glucose (mg/dL)	159.54 ± 7.74	130.08 ± 6.58[b]	131.02 ± 3.63[b]	(↓ 17.8%)
Postprandial glucose (mg/dL)	248.30 ± 11.82	196.48 ± 11.23[b]	183.54 ± 10.54[b]	(↓ 26.0%)
HbA1c (%)	7.22 ± 0.14	6.65 ± 0.14[b]	6.41 ± 0.11[b]	(↓ 11.2%)

Data are expressed as mean ± SEM; [b]$P < 0.001$ compared to 0[th] d levels.

Table 2 Effect of 6 mo polyherbal therapy on lipidemia

Biochemical parameter	Variations at 3 monthly intervals following polyherbal therapy			
	0 d	3 mo	6 mo	Mean change
Total cholestero (mg/dL)	162.08 ± 6.00	144.84 ± 5.20[b]	146.8 ± 4.70[a]	(↓ 9.4%)
Triglyceride (mg/dL)	140.81 ± 6.88	126.74 ± 6.88	122.3 ± 4.50[a]	(↓ 13.1%)
HDL cholesterol (mg/dL)	34.38 ± 1.37	35.28 ± 1.04	37.9 ± 1.20[a]	(↑ 9.8%)
LDL cholesterol	99.33 ± 5.61	84.21 ± 5.21[b]	81.98 ± 4.84[a]	(↓ 17.4%)
VLDL (mg/dL)	28.16 ± 1.37	25.35 ± 1.37	23.71 ± 1.13[a]	(↓ 15.8%)

Data are expressed as mean ± SEM; [a]$P < 0.05$, [b]$P < 0.001$ compared to 0[th] d levels. HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein.

and was provided by M/S Deendayal Aushadhi Pvt. Ltd., India. Each subject had 50 mL of fresh hot water extract derived from 10 gm of "Diabegon" powder soaked overnight in water administered daily on an empty stomach and therapy continued for six months without any break under the supervision of an Ayurvedic physician. The study design was approved by the Institutional Human Ethics Committee of Jiwaji University.

Biochemical parameters

The fasting and postprandial plasma glucose measurements were determined at monthly intervals, while the glycosylated hemoglobin, antioxidant parameters such as super oxide dismutase, catalase, glutathione (GSH), Thiobarbituric Acid Reactive Substances (TBARS) and lipid profile, functional markers of kidney and liver function were monitored at baseline, at the middle (3 mo) and at the end (6 mo) of the therapy.

Fasting and postprandial plasma glucose was estimated by the Glucose oxidase/Peroxidase method[19]. Glycosylated hemoglobin (HbA1c) was estimated by the ion exchange resin method[20]. Estimation of plasma total cholesterol by the Cholesterol oxidase - Phenol-aminophenazone (CHOD-PAP) method[21], triglyceride by the GPO-PAP method[22], HDL by the Polyethylene glycol/ Cholesterol oxidase-Phenol-aminophenazone Polyethylene glycol/CHOD-PAP method[23], LDL and VLDL were calculated by the Friedewald formula, urea by the modified Berthelot method[24], uric acid by the uricase/PAP method[25], creatinine by modified Jaffe's kinetic method[26], serum glutamate pyruvate transaminase (SGPT or alanine transaminase) and serum glutamate oxaloacetate transaminase (SGOT or AST) by the modified International Federation of Clinical Chemistry method[27] and bilirubin[28] was assayed using standard kits from Crest Biosystems, Goa (India). Superoxide dismutase and catalase activities were assayed by Winterbourn *et al*[29] and by Sinha *et al*[30] respectively. Estimation of reduced GSH and TBARS was done by the method of Ellman[31] and Ohkawa *et al*[32] respectively, and protein was estimated by the method of Lowry *et al*[33], estimation of urinary sugar by Benedict's method[34] and urinary protein by the sulfosalicylic method[35].

Ethical clearance

The study protocol was duly approved by the Institutional Human Ethics Committee (JU/IHEC/2013-A/10).

Statistical analysis

Statistical analysis was done by a paired *t* test (Sigma stat 3.5).

RESULTS

Effect of polyherbal therapy on blood glucose levels

Table 1 shows the fasting and postprandial blood glucose levels at 3 monthly intervals following polyherbal therapy. A significant decrease ($P < 0.001$) was recorded in both fasting and postprandial glucose levels (17.8% and 26% respectively) and glycosylated hemoglobin (11.2%) at the end of six months therapy.

Effect of polyherbal therapy on lipidemia

Table 2 shows the results of the lipid profile. Total cholesterol, triglycerides, LDL and VLDL significantly decreased by 9.4%, 13.1%, 17.4%, 15.8% respectively ($P < 0.05$). HDL cholesterol significantly increased from 34.38 ± 1.37 to 37.78 ± 1.26 ($P < 0.05$) at the end of the therapy.

Effect of polyherbal therapy on biomarkers of oxidative stress

Significant ($P < 0.05$), improvements in GSH level (from 2.29 ± 0.26 to 3.03 ± 0.12 mg/dL), SOD activity (from 0.47 ± 0.07 to 0.74 ± 0.04 μmol/L min^{-1} per mg protein), catalase activity (from 4.19 ± 0.37 to 6.07 ± 0.23 μmol/L min^{-1} per milligram protein) and levels of TBARS (from 486.62 ± 29.82 to 442.26 ± 21.44 (moles of Malondialdehyde/mL of blood) were recorded at the end of polyherbal therapy (Table 3).

Effect of polyherbal therapy on biomarkers of toxicity

The effect of polyherbal therapy on kidney function was monitored by estimating urea, creatinine and uric acid levels in plasma at various intervals during the course of therapy. The data presented in Table 4 showed significant reductions in uric acid (from 5.35 ± 0.21 to 4.80 ± 0.97 mg/dL) and creatinine (from 0.78 ± 0.04 to 0.60 ± 0.02 mg/dL) and there was no significant change in the level

Table 3 Effect of 6 mo polyherbal therapy on biomarkers of oxidative stress

Biochemical parameter	Variations at 3 monthly intervals following polyherbal therapy			
	0 d	3 mo	6 mo	Mean change
GSH (mg/mL)	2.29 ± 0.26	2.74 ± 0.14	3.03 ± 0.12[a]	(↑ 32.3%)
SOD (μmol/L·min^{-1} per milligram protein)	0.47 ± 0.07	0.58 ± 0.03	0.74 ± 0.04[a]	(↑ 57.4%)
Catalase (μmol/L·min^{-1} per milligram protein)	4.19 ± 0.37	4.70 ± 0.27	6.07 ± 0.23[b]	(↑ 44.8%)
TBARS (*n* moles of MDA/mL of blood)	486.62 ± 29.82	455.11 ± 21.81[a]	442.26 ± 21.44[a]	(↓ 9.1%)

Data are expressed as mean ± SEM; [a]$P < 0.05$ compared to 0[th] d levels. GSH: Glutathione; SOD: Superoxide dismutase; TBARS: Thiobarbituric acid reactive substances; MDA: Malondialdehyde.

Table 4 Effect of 6 mo polyherbal therapy on biochemical markers of kidney function

Biochemical parameter	Variations at 3 monthly intervals following polyherbal therapy			
	0 d	3 mo	6 mo	Mean change
Urea (mg/dL)	26.31 ± 0.97	26.45 ± 0.90	25.48 ± 0.87	(↓ 3.1%)
Uric acid (mg/dL)	5.35 ± 0.21	5.36 ± 0.16	4.80 ± 0.97[a]	(↓ 10.2%)
Creatinine (mg/dL)	0.78 ± 0.04	0.76 ± 0.02	0.60 ± 0.02[b]	(↓ 23.0%)

Data are expressed as mean ± SEM; [a]$P < 0.05$, [b]$P < 0.001$ compared to 0[th] d levels.

of urea.

Significant variations in enzyme markers of liver, namely SGOT (from 24.00 ± 3.04 to 21.49 ± 1.67 IU/L) and SGPT (from 25.33 ± 3.27 to 21.83 ± 64 IU/L), were recorded (Table 5). There was no change in the level of bilirubin.

Effect of polyherbal therapy on hypertension and body mass index

Table 6 shows variations in systolic blood pressure (130.40 ± 2.27 to 126.12 to 2.41 mmHg), diastolic blood pressure (81.06 ± 1.14 to 77.90 ± 1.35 mmHg) and body mass index (from 25.75 ± 0.57 to 24.85 ± 0.50 kg/m^2).

Effect of polyherbal therapy on glycosuria and proteinuria

There were significant reductions in urinary sugar (64%) and urinary protein levels (60%) (Table 7) following polyherbal therapy.

DISCUSSION

The majority of the formulations used in Ayurveda are based on herbs and used as decoctions, infusion, tinctures and powders. The decoction of polyherbal formulation used in the present study (named "Diabegon kwath" in Ayurvedic terminology) contained hot water extract of powdered plant parts of *Gymnema sylvestre* (*Gurmar*), *Eugenia jambolana* (*Jamun seed*), *Emblica officianale* (*Amla*), *Curcuma longa* (*Haldi*), *Pterocarpus marsupium* (*Vijaysaar*), *Terminalia chebula* (*Harad*) *Cassia fistula* (*Amaltas*), *Picrorhiza kurroa* (*Kutki*), *Swertia charita* (*Chiraita*) and *Terminalia bellerica* (*Behada*) in varying amounts. Administration of "Diabegon kwath" over a period of 6 months to type Ⅱ diabetic subjects with varying degrees of hyperglycemia and hyperlipidemia resulted in significant alleviation of these metabolic abnormalities. Marked improvements in glucose homeostasis, as evident from significant changes in glycosylated hemoglobin and blood glucose levels, and lipid profile, as evident from elevations in HDL choles-

Table 5 Effect of 6 mo polyherbal therapy on biochemical markers of liver function

Biochemical parameter	Variations at 3 monthly intervals following polyherbal therapy			
	0 d	3 mo	6 mo	Mean change
Total bilirubin (mg/dL)	0.98 ± 0.05	0.91 ± 0.06	0.97 ± 0.04	(↓ 1.0%)
SGOT (IU/L)	24.00 ± 3.04	21.70 ± 2.04[a]	21.49 ± 1.67[a]	(↓ 10.4%)
SGPT (IU/L)	25.33 ± 3.27	22.70 ± 2.24[a]	21.83 ± 1.64[a]	(↓ 13.8%)

Data are expressed as mean ± SEM; [a]$P < 0.05$ compared to 0[th] d levels. SGOT: Serum glutamate oxaloacetate transaminase; SGPT: serum glutamate pyruvate transaminase.

Table 6 Effect of 6 mo polyherbal therapy on blood pressure and anthropometry

	Variations at 3 monthly intervals following polyherbal therapy			
	0 d	3 mo	6 mo	Mean change
Systolic blood pressure (mmHg)	130.40 ± 2.27	125.15 ± 2.09[a]	126.12 ± 2.41[a]	(↓ 3.2%)
Diastolic blood pressure (mmHg)	81.06 ± 1.14	78.97 ± 1.23	77.90 ± 1.35	(↓ 3.8%)
Body mass index (kg/m^2)	25.75 ± 0.57	25.19 ± 0.56[b]	24.85 ± 0.50[b]	(↓ 3.4%)

Data are expressed as mean ± SEM; [a]$P < 0.05$, [b]$P < 0.001$ compared to 0[th] d levels.

Table 7 Effect of 6 mo polyherbal therapy on glycosuria and proteinuria

Parameter	Variations at 3 monthly intervals following polyherbal therapy			
	0 d	3 mo	6 mo	Mean change
Urinary sugar (gm/dL)	0.74 ± 0.10	0.34 ± 0.06[b]	0.27 ± 0.06[b]	(↓ 63.5%)
Urinary protein (mg/dL)	63.33 ± 16.68	43.63 ± 15.15	25.45 ± 6.50[a]	(↓ 59.8%)

Data are expressed as mean ± SEM; [a]$P < 0.05$, [b]$P < 0.001$ compared to 0[th] d levels.

terol with concomitant decreases in other lipids, were observed. One of the major ingredients of the polyherbal preparation studied is *Gynmnema Sylvestre* which is reported to promote insulin secretion, probably by regeneration of pancreatic beta cells[36]. *In vitro* trials on experimental models with *Gynmnema Sylvestre* have proved that this herbal drug increases insulin release by increasing the cell permeability[37]. The *G. sylvestre* is reported to inhibit absorption of glucose from intestine. The leaves of *G. sylvestre* contain gymnemic acid and the atomic arrangement of gymnemic acid molecules is similar to that of glucose molecules. Gymnemic acid molecules fill the receptor location in the absorptive external layers of the intestine, thereby preventing the sugar molecules absorption by the intestine, which ultimately results in low blood sugar level[38]. *Pterocarpus marsupium* is effective in reducing levels of blood glucose and glycosylated hemoglobin in type 2 diabetic patients[39]. Alcoholic extract of *Picrorrhiza kurroa* (75 mg extract/kg) reduced serum glucose that was at a maximum 2 h after the dose. It also showed an antihyperglycemic effect in alloxanized diabetic rats. Serum glucose decreased by 43% and 60% with 75 and 150 mg/kg of the extracts, respectively. Antioxidant activity is also described in the literature[40]. Hexane fraction of *Swertia chirayita* at 250 mg/kg, *po* to normal rats significantly reduced blood sugar and increased plasma insulin without influencing hepatic glycogen content. However, when administered for 28 d, it significantly increased hepatic glycogen content in conjunction with other effects, probably by releasing insulin[41]. Decoction of stem bark of *Cassia fistula Linn.* improved glucose tolerance, significantly inhibited the glucose absorption from the small intestine and provoked glycogen accumulation in liver and skeletal muscle[42,43]. *Terminalia chebula* exhibited *in vitro* antioxidant and free radical-scavenging activities[44]. The antihyperglycemic effect of *T. chebula* is due to its ability to restore the functions of pancreatic tissues by causing an increase in insulin output, inhibiting the intestinal absorption of glucose or facilitating the metabolites in insulin dependent processes. In India, decoction of kernels of *Eugenia jambolana* is used as household remedy for diabetes. The antihyperglycemic effect of aqueous and alcoholic extract as well as lyophilized powder showed a reduction in blood glucose level[45]. Hence, treatment with herbal drugs has an effect on protecting β-cells and smoothing out fluctuation in glucose levels[46,47].

Studies were conducted earlier with polyherbal formulations with varying contents and compositions for their antihyperglycemic potential. A polyherbal formulation, Dihar[18], containing eight different herbs, *Syzygium cumini*, *Momordica charantia, Emblica officinalis*, *Gymnema sylvestre*, *Enicostemm*, *Azadirachta indiaca*, *Tinospora cordifolia and Curcuma longa*[18], showed effective antihyperglycemic activity in streptozotocin (STZ, 45 mg/kg *iv* single dose) induced diabetes in rats. A polyherbal formulation, termed DRF/AY/5001, containing *Gymnema sylvestre*, *Syzygium cumini*, *Pterocarpus marsupium*, *Momordica charantia*, *Emblica officinalis*, *Terminalia belirica*, *Terminalia chebula* and *Shudh shilajit*, showed an antihyperglycemic effect similar to Glibanclamide[48]. Similarly, a polyherbal formulation, namely "Diabecon", containing *Gymnema sylvestre*, *Pterocarpus marsupium*, *Glycyrrhiza glabra*, *Casearia esculenta*, *Syzygium cumini*, *Asparagus racemosus*, *Boerhavia diffusa*, *Sphaeranthus indicus*, *Tinospora cordifolia*, *Swertia chirata*, *Tribulus terrestris*, *Phyllanthus amarus*, *Gmelina arborea*, *Gossypium herbaceum*, *Berberis aristata*, *Aloe vera*, *Triphala*, *Commiphora wightii*, *shilajeet*, *Momordica charantia*, *Piper nigrum*, *Ocimum sanctum*, *Abutilon indicum*, *Curcuma longa* and *Rumex maritimus*, is reported to increase peripheral utilization of glucose, increase hepatic and muscle glucagon contents, promote B-cells repair and regeneration, and increase C-peptide level. It exhibited antioxidant properties and protected β-cells from oxidative stress. "Glyoherb" granules were shown to possess potential antidiabetic activity, lowered serum glucose levels and increased glucose tolerance in STZ-induced type 1 diabetic rats. This polyherbal formulation also possesses significant antihyperlipidemic activity as it lowered serum cholesterol and triglyceride levels. "Glyoherb" did not exert any toxic effects in STZ-induced impaired kidney and liver functions. It was found rather to improve kidney and liver functions. In addition, "Glyoherb" possesses potential antioxidant activity as it decreases lipid peroxidation and enhances antioxidant status in diabetic rats[49,50] and it was reported that the treatment with *Coccinia cordifolia* extract of newly detected type 2 diabetic patients for 90 d results in a 16% decrease in fasting blood glucose level and 18% in PP blood glucose level. Several studies of medicinal plants claimed to have a significant reduction in blood glucose level but in the present study, HbA1C percentage was significantly decreased in type Ⅱ diabetes subjects after 6 mo of treatment, suggesting that there is a reduction of generalized glycosylation of proteins in circulation. A significant reduction in glycosuria and proteinuria was observed in type Ⅱ diabetic subjects on "Diabegon kwath" therapy.

Oxidative stress plays a major role in the pathogenesis of both types of diabetes mellitus. Free radical production caused by hyperglycemia may occur by at least three different routes: nonenzymatic glycation[51], auto-oxidation of glucose and intracellular activation of the polyol pathway[52,53]. High levels of free radicals and simultaneously declined antioxidant enzyme levels lead to cell damage, inactivation of enzymes and lipid peroxidation. The "Diabegon kwath" in the present study also exhibited potent antioxidant activity, as evident from restoration of the activities of antioxidant enzymatic activities studied. The *Emblica officinalis* which is a rich source of vitamin C has been reported to reduce lipidemia and free radical production in experimental animals, considered to be the most important causative factors for diabetes-related complications. The *E. officinalis* and its enriched tannoids delay diabetic cataract in rats[54]. The lipid levels, such as cholesterol and triglycerides, in serum and liver were markedly elevated in aged control rats, while they were significantly decreased by the administration of amla[55]. There is an increased quest to obtain natural antioxidants

with broad spectrum actions. The herbal formulation used in the present study shows significant improvement in markers of oxidative stress, besides antihyperglycemic and antihyperlipidemic functions. Furthermore, oral administration of "Diabegon kwath" daily for 6 mo had no adverse effects, either on kidney or liver functions and in fact a marked improvement in functioning of these vital organs was noticed.

In conclusion, the present study with "Diabegon kwath" in type Ⅱ diabetic subjects with varying degrees of hyperglycemia, hyperlipidemia and oxidative stress proved that the formulation serves as an effective alternative to conventional antidiabetic therapies. Furthermore, the formulation was found to improve liver and kidney functions and may be regarded as a promising natural and safe remedy for the prevention of diabetic complications. This is the first long term study with any polyherbal formulation in human type Ⅱ diabetes mellitus.

ACKNOWLEDGMENTS

We thank Deendayal Aushadhi Pvt. Ltd., Gwalior (M.P.) for the supply of the polyherbal formulation and the financial support from AYUSH, New Delhi, is duly acknowledged. We are grateful to all the human subjects who volunteered to participate in the study. Our profound thanks are due to Dr. Mohit Tiwari, Dr. Sheetal Gupta of M/S Deendayal Aushadhi Pvt. Ltd., Gwalior, Sri Dinesh Borole, other colleagues of the laboratory and students for their unreserved support in conducting this study.

COMMENTS

Background

Type 2 diabetes is a multifaceted lifelong disorder and can lead to micro and macrovascular complications when left unchecked. The oral hypoglycemic agents available for the treatment of type II diabetes mellitus are reported to exhibit undesired side effects in a considerable number of subjects, even under euglycemic conditions. Polyherbal preparations are shown to function as potential antihyperglycemic agents.

Research frontiers

Polyherbal based Ayurvedic formulations are in regular use in southeastern countries as drug supplements for the treatment of type II diabetes mellitus. Scientific validation of such preparations and their safety evaluations are major concerns to biomedical scientists working on indigenous systems of medicine.

Innovations and breakthroughs

Several short term studies in experimental models revealed antidiabetic potentials of many polyherbal based formulations and very few herbal drug preparations have succeeded in human trials. This is the first ever long term open study validating an antidiabetic Ayurvedic formulation in human type II diabetes mellitus. The study not only evaluated the efficacy of the Ayurvedic polyherbal formulation but also addressed the safety concerns in human subjects.

Applications

The study revealed that "Diabegon kwath" functions as an effective alternative antidiabetic drug formulation which can safely be advocated for treatment of human type 2 diabetes mellitus.

Terminology

"Diabegon kwath" is a hot water extract of defined plant/herb parts.

Peer review

The study is interesting and not well known in the majority of countries. This is a long term study about the efficacy of "Diabegon kwath", a polyherbal formulation with diverse therapeutic functions. The formulation acts on glucose metabolism, lipids and functions as an antioxidant and the results of the study are significant.

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World Journal of Translational Medicine

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ISSN 2220-6132 (online)

Launch date

June 12, 2012

Frequency

Four-monthly

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