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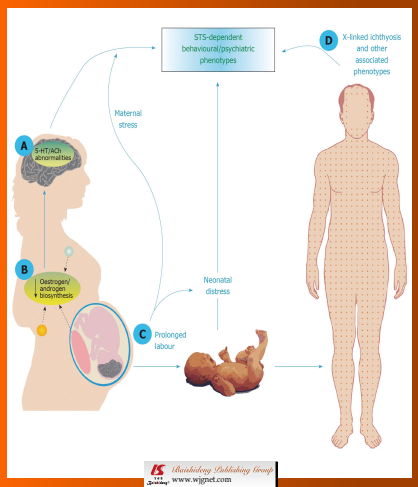

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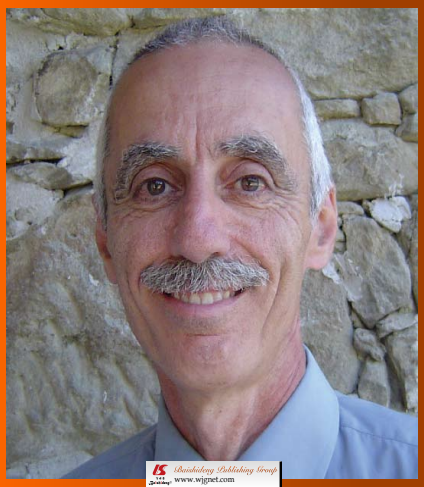

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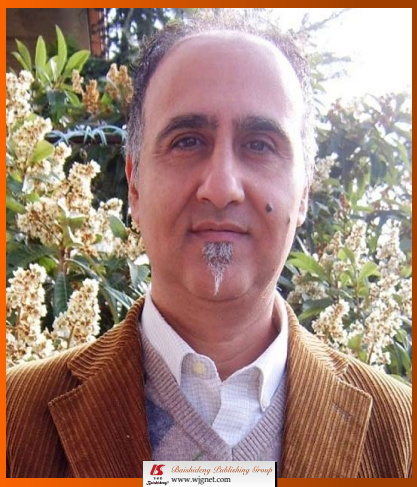

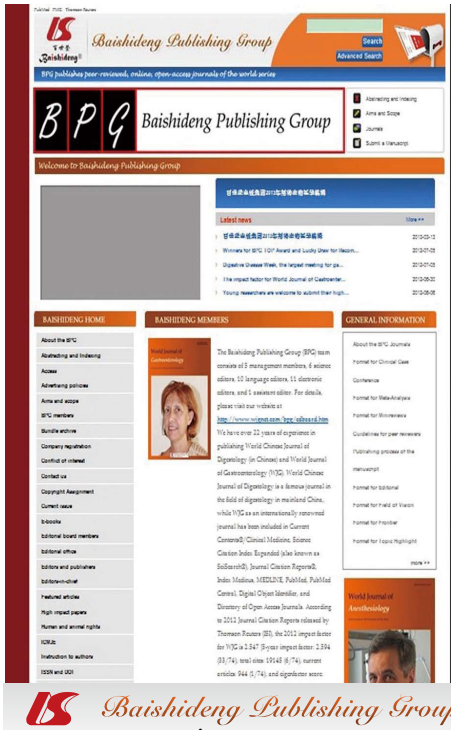

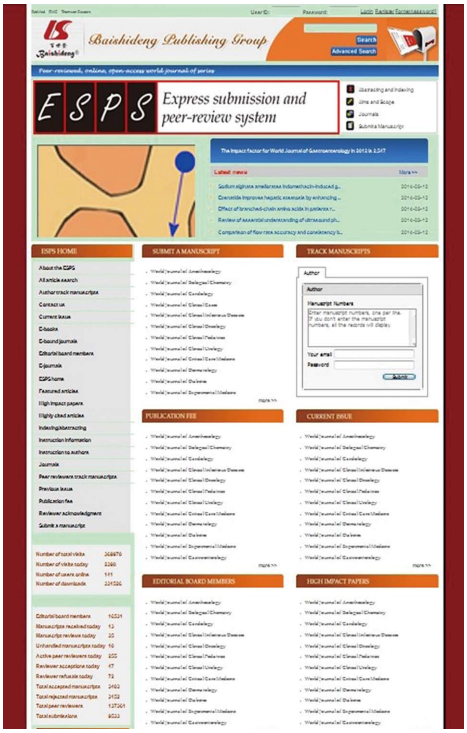

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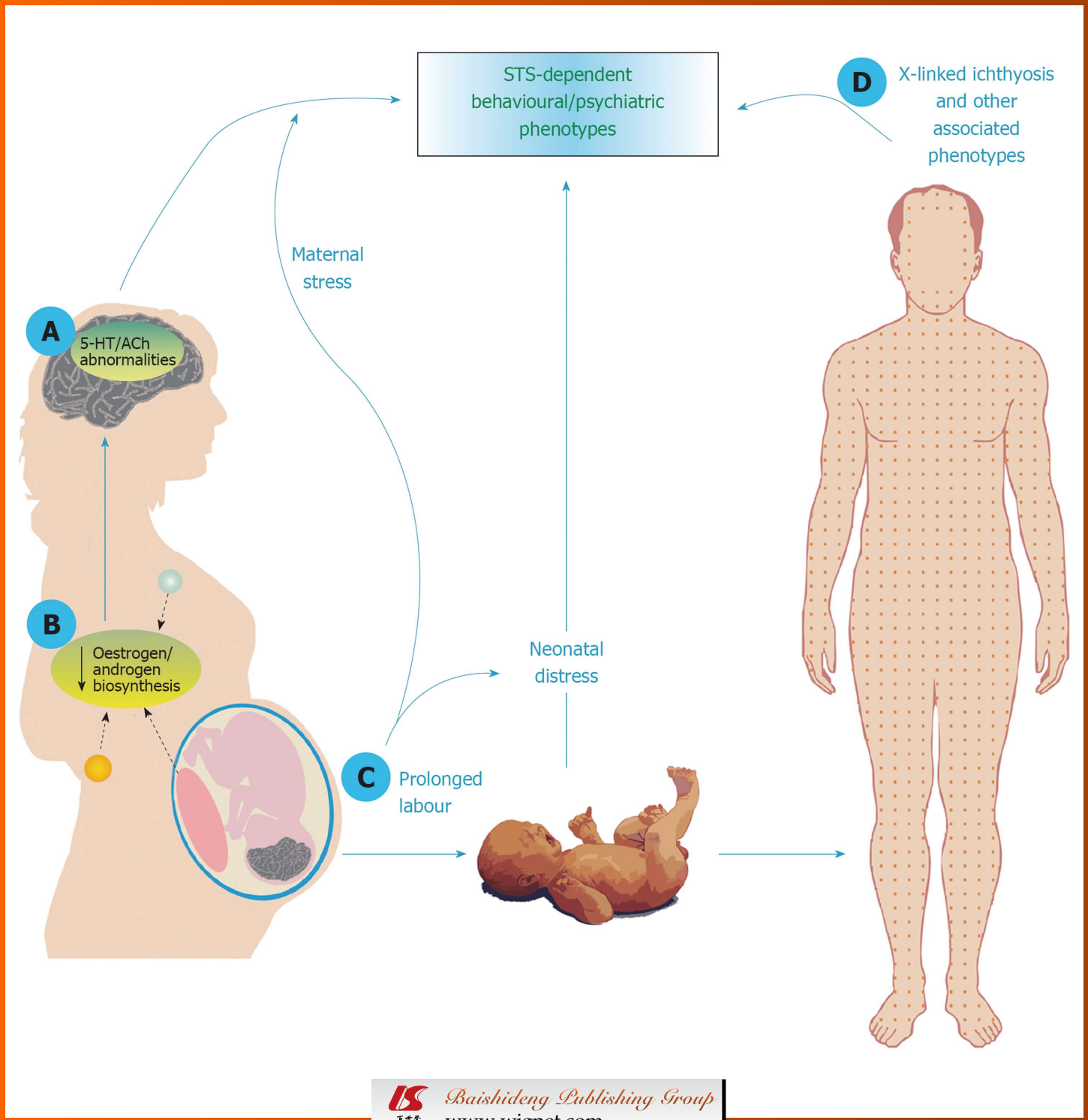

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REVIEW

1 Cognitive, behavioural and psychiatric phenotypes associated with steroid sulfatase deficiency

Trent S, Davies W

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REVIEW

Cognitive, behavioural and psychiatric phenotypes associated with steroid sulfatase deficiency

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Abstract

The enzyme steroid sulfatase (STS) desulfates a variety of steroid compounds thereby altering their activity. STS is expressed in the skin, and its deficiency in this tissue has been linked to the dermatological condition X-linked ichthyosis. STS is also highly expressed in the developing and adult human brain, and in a variety of steroidogenic organs (including the placenta and gonads); therefore it has the potential to influence brain development and function directly and/or indirectly (through influencing the hormonal milieu). In this review, we first discuss evidence from human and animal model studies suggesting that STS deficiency might predispose to neurobehavioural abnormalities and certain psychiatric disorders. We subsequently discuss potential mechanisms that may underlie these vulnerabilities. The data described herein have potential implications for understanding the complete spectrum of clinical phenotypes associated with X-linked ichthyosis, and may indicate novel pathogenic mechanisms underlying psychological dysfunction in developmental disorders such as attention deficit hyperactivity disorder and Turner syndrome.

Key words: Acetylcholine; Aggression; Attention; Attention deficit hyperactivity disorder; Dehydroepiandrosterone sulfate; Impulsivity; Hippocampus; Postpartum psychosis; Serotonin

Core tip: The enzyme steroid sulfatase (STS) cleaves sulfate groups from neuroactive steroid hormones thereby altering their activity. Here, we review cross-species evidence indicating that deficiency for this enzyme might influence behaviour and vulnerability to psychiatric illness; we then suggest potential mediating mechanisms. Understanding whether or not STS deficiency impacts upon neural function, and if so, how, has potential implications for diagnosis, counselling and treatment in cases of X-linked ichthyosis (the dermatological condition associated with STS deficiency). Moreover, this understanding may provide more general novel insights into the pathogenesis of common and disabling psychiatric disorders.

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STEROID SULFATASE AND ITS ROLE IN BRAIN FUNCTION

Steroid sulfatase (STS, formerly known as arylsulfatase C) is an enzyme that acts as a homodimer within the endoplasmic reticulum to cleave sulfate groups from a variety

of sulfated steroid hormones (notably 16α-hydroxy-dehydroepiandrosterone, dehydroepiandrosterone, estrone and cholesterol sulfates), thereby altering their biological function; the desulfation of several of these compounds represents an initial step in the biosynthesis of a number of androgens and oestrogens[1]. The STS protein is expressed in a number of tissues important in reproductive function including the placenta (highest expression), the ovaries, the testes and the mammary gland, as well as other non-reproductive tissues such as the liver and thyroid gland[2] (http://www.ncbi.nlm.nih.gov/unigene).

Besides potentially influencing the development and/or ongoing function of the aforementioned organs, STS represents an excellent *a priori* candidate modulator of brain development and behaviour: during human embryogenesis, *STS* expression occurs throughout the thalamus, in the cortical plate, throughout the basal ganglia, in the hypothalamus and anterior pituitary gland, and in the cerebellar neuroepithelium (a pattern largely consistent with that seen in other mammalian and non-mammalian species)[3], and with reported sulfatase activities in *post mortem* brain tissue of adult humans[4]. Given this persistent brain expression, STS is likely to influence neural function directly; the substrates and products of the enzyme are known to act as modulators at key sites of neurotransmission in the brain, notably at γ aminobutyric acid type A, N-methyl-D-aspartic acid and sigma (σ)receptors[1]. In addition, STS could exert a significant indirect influence on the brain *via* its role in androgen and oestrogen biosynthesis; these compounds can act systemically either to substantially and permanently alter brain development ("organisational effects") or to influence ongoing neural function *via* more subtle, potentially reversible "activational effects"[5].

In man, STS is encoded by the X-linked *STS* gene, which resides just outside pseudoautosomal region (PAR) 1 at Xp22.3. *STS* escapes X-inactivation[6], and its Y homologue is pseudogenic as a consequence of a pericentric inversion[7]. Together these attributes suggest the possibility that the gene might be expressed more highly in female than male tissues, and hence the activity of the associated enzyme may be greater in the former sex; there is some empirical evidence that this may be the case[8-10]. A recent expression analysis has suggested that alternative first exons of the *STS* gene may be employed in different tissues, with exons 0a and 1b being most abundant in the brain[11]. In rats, as in man, *STS* is X-linked, but the rat orthologue appears to be subject to X-inactivation[12]. In mice, *STS* is located within the PAR, and therefore by definition, escapes X-inactivation[13]. At the genetic level, there is considerable sequence divergence across species, although the function of the encoded enzyme appears to be largely conserved[12,13].

STS DEFICIENCY AND EFFECTS ON GENERAL PHYSIOLOGY

The vast majority of cases of STS deficiency (85%-90%) are caused by complete/partial deletions of the *STS* gene, which typically also encompass genes immediately adjacent (*HDHD1A*, *PNPLA4* and *VCX*); larger, rarer deletions may encompass more distant genes including *ARSE* (encoding arylsulfatase E), *NLGN4X* (neuroligin 4X), other members of the *VCX* family (encoding variably charged proteins) and *KAL1* (encoding anosmin-1)[14-21]. About 5%-10% of patients with STS deficiency present with a complex phenotype arising from the deletion of one or more of the disease genes listed above: altered neuroligin 4X function has previously been suggested to account for sporadic cases of autism and mental retardation[20,22,23], deficiency for the *VCX* and *VCX3A* genes has been suggested as a possible cause of mental retardation[24-26] (although there is some evidence that deletion of these genes alone is not sufficient to cause this phenotype[27-29]), arylsulfatase E dysfunction has been linked to the skeletal disorder chondrodysplasia punctata[30], and mutations within *KAL1* (encoding anosmin-1) are associated with Kallman syndrome characterised by hypogonadotropic hypogonadism and anosmia[31]. The remaining 10% of cases of STS deficiency may be caused by a either variety of point mutations within the *STS* gene resulting in aberrant gene expression/splicing or protein function[32-39], or, rarely, by a deleterious mutation in the autosomal gene encoding sulfatase modifying factor-1 (*SUMF1*) whose normal role is the posttranslational modification and catalytic activation of a host of sulfatase enzymes[40]. The majority of genetic mutations at the *STS* locus are likely to be inherited rather than arising *de novo*[41].

The main phenotype associated with STS deficiency is a comparatively benign dermatological condition (X-linked ichthyosis, XLI) in which affected individuals present with large dark brown, adherent scales (particularly on the trunk, arms and legs) as a consequence of the accumulation of cholesterol sulfate in the membranes of stratum corneum cells[42]. Unsurprisingly, given that *STS* is X-linked, the vast majority of subjects diagnosed with XLI are male, although skin dryness may be manifest in female heterozygotes. Other, less common, phenotypes associated with STS deficiency include corneal opacities which do not affect vision (10%-50% of cases), and maldescent of the testes during embryogenesis (20% of cases)[42]. Expectant mothers carrying fetuses affected by STS deficiency tend to exhibit delayed or prolonged labour as a consequence of reduced placental oestrogen production and insufficient cervical dilation[43].

Confirmation of STS deficiency in cases ascertained clinically (generally through their skin condition) may be done biochemically through showing absent enzyme activity, or genetically through identifying either a complete/partial *STS* deletion or a deleterious point mutation within the gene[42]. Prevalence estimates for XLI based on clinical ascertainment have been in the range of 1 in 3000-6000 males; however, prenatal screens identifying cases of STS deficiency through low levels of maternal serum oestrogen (unconjugated estriol) have reported higher prevalences of approximately 1 in 1500 males[44,45].

These data indicate a spectrum of phenotypic consequences of enzyme deficiency, ranging from the most severe and easily ascertained (including contiguous gene syndromes), to milder forms (skin abnormalities only), to forms with no obvious clinical implications.

As the *STS* locus escapes X-inactivation, loss of genetic material from the short arm of the X chromosome, or loss of an entire X chromosome, in females as occurs in the developmental disorder Turner syndrome (TS)[46], will result in haploinsufficiency for STS. Whilst such haploinsufficiency is unlikely to result in phenotypes as obvious as those caused by complete enzyme deficiency, it may still feasibly contribute to the physiological and psychological abnormalities seen in TS[47]. Moreover, loss of one *STS* allele in TS could expose deleterious mutations on the remaining allele.

STS DEFICIENCY: EFFECTS ON BEHAVIOUR AND VULNERABILITY TO PSYCHIATRIC DISORDER

Possible role in disorders of attention

Emerging data from a variety of experimental sources is providing converging evidence for a role for STS in the modulation of attention. In the first systematic study of behaviour in individuals with XLI, Kent *et al*[20] showed that within a sample of 25 affected boys, ten met DSM-IV criteria for diagnosis of attention deficit hyperactivity disorder (ADHD), a neurodevelopmental condition characterised by inattention, pathological impulsivity and hyperactivity[48]; crucially, this sample was originally ascertained on the basis of low unconjugated estriol levels in their pregnant mothers and not on the basis of the boys' behaviour. Of the ten individuals affected by ADHD, eight were diagnosed with primarily inattentive subtype of the disorder, whilst the remaining two were diagnosed with the combined subtype (exhibiting evidence of inattention, and impulsivity and/or hyperactivity). Although this study did not employ a matched-control group, the 40% overall ADHD diagnosis rate (and 32% inattentive ADHD diagnosis rate) within the XLI group was substantially higher than that typically observed within the United Kingdom general population (4%-6% overall, 0.5% inattentive). Importantly, whilst most boys diagnosed with inattentive ADHD had deletions spanning multiple genes, two individuals within this subgroup had presumed inactivating point mutations within *STS*, indicating that STS dysfunction *per se* might predispose to inattention rather than the lack of gene product from a contiguous gene. The findings of the Kent *et al*[20] study are consistent with previous, more limited, case reports in the literature that have described individuals with contiguous Xp22.3 gene deletions and ADHD[49-51]. These initial data indicate that genetic screening of large, behaviourally-ascertained ADHD samples and appropriate control samples to investigate the relative prevalence of *STS* deletions/point mutations may be worthwhile.

Whilst work stimulated by the initial XLI findings has indicated no significant association between polymorphisms within *STS* and overall ADHD risk after correction for multiple testing[3,11], there does appear to be a significant, and replicable, association between variation at rs17268988 (located within intron 9 of *STS*) and number of inattentive symptoms within ADHD cohorts[3,52]; specifically, possession of the minor G allele at this site (allele frequency about 0.25) is associated with a greater number of inattentive symptoms, particularly in older children (> 9 years of age). Whilst the genetic, cellular and neural mechanisms through which this association is mediated remain obscure, this finding provides further evidence for a role of STS in attentional function in neurodevelopmentally-compromised subjects; the extent to which an association between this genetic variant and attention exists in healthy individuals remains to be tested. Other polymorphisms across the *STS* gene (rs12861247, rs5978405 and rs5933863) have been shown to be significantly associated with aspects of cognitive function in males with ADHD as indexed by their performance on the comprehension, verbal IQ and picture completion Wechsler subtests, respectively[3]; as a small sample size was employed in this study these findings could be spurious, but if confirmed, these associations could feasibly also be mediated *via* effects on attention.

One of the most consistently reported neuropsychological findings in women with TS is inattention[53] which can be manifest as heightened distractability in real-life situations[54]. Rates of ADHD have been reported to be up to eighteen-fold higher in the TS population than in a control female population[55]. By correlating individual TS subjects' aggregate cognitive scores (partly based upon measures of attention) with their karyotype, Zinn *et al*[56] concluded that haploinsufficiency for an 8.3Mb region of chromosome Xp22.3 housing just 31 annotated genes (including *STS*) was critical in the development of the characteristic TS cognitive profile. Given the results arising from the XLI and ADHD studies described above, *STS* is a candidate for the attentional component of this profile. As such, it will be interesting to test whether those subjects with TS at greatest risk of attention deficits are hemizygous for the previously-identified risk alleles or deleterious mutations within *STS*. Should this prove to be the case, it would offer an opportunity to provide better genetic counselling and earlier clinical intervention in cases of TS.

Attention deficits are a prominent clinical feature of neuropsychiatric disorders other than ADHD, including autism[57-59] and schizophrenia[60]; cytogenetic deletions encompassing *STS* have been reported in individuals affected by both disorders[20,61-65]. Psychiatric disorders associated with attention problems are more common (*e.g.,* ADHD and autism[66]) or more severe (*e.g.,* schizophrenia[67,68]) in males than in females. Thus, it is plausible that the lower expression/activity of STS in males reduces their threshold of vulnerability to attentional dysfunction.

Recent data from animal model work appears to

substantiate the link between STS dysfunction and inattention. Performance deficits in the 39, XO mouse (a model of TS[69]) on the 5-choice serial reaction time task (5-CSRTT) assaying visuospatial attention could be rescued by the addition of a small chromosome containing a small number of additional genes including *STS*[70]. Subsequent work in another genetic model, the 39, $X^{Y*}O$ mouse (in which the *STS* gene is deleted as a consequence of an end-to-end fusion of the X and Y chromosomes within the PAR), revealed that these mice are less able to detect stimuli of short duration than wildtype mice[71]. Parallel studies in mice in which the STS axis was acutely pharmacologically modulated also showed effects on attention; specifically, administration of the enzyme substrate dehydroepiandrosterone sulfate (DHEAS) enhanced a main index of attention, whilst administration of the specific enzyme inhibitor COUMATE impaired response accuracy under attentionally-demanding conditions[71]. These pharmacological data, besides hinting that brain DHEA(S) levels may be a pertinent factor in attentional function, also indicate that ongoing STS activity could influence this psychological process. Given that peripherally-administered DHEAS is rapidly converted to DHEA within the mammalian brain[72], it is plausible that high levels of the latter compound within the brain are associated with enhanced attention, but that low levels (as presumably occur in XLI patients and 39, $X^{Y*}O$ mice) are associated with impaired attention. Whilst animal model work has provided some preliminary clues as to brain pathways that might be affected by STS and hence which might underpin its effect on attention (see later), more in-depth analyses are clearly required. As STS appears to influence ongoing attentional processes, such analyses may feasibly identify novel therapeutic targets that could be acutely pharmacologically modulated in adolescents and adults affected by disorders of attention.

Aggression

Early genetic evidence in mice examining inter-male aggression indicated that the Y chromosome PAR played an important role[73]; in mice, the PAR was originally thought to house just one gene (*STS*), but recently a second mouse PAR gene *Asmt* (encoding the enzyme acetylserotonin O-methyltransferase involved in the biosynthesis of melatonin from serotonin) has been identified[74]. However, in support of STS as a candidate mediator of this phenotype, a strong relationship between protein levels and aggression has been noted across several inbred mouse strains[75] and co-administration of both DHEAS and COUMATE resulted in heightened aggression in the inbred CBA/H strain[76]; this latter result suggests that, in addition to modulating ongoing attentional function in mice, STS may also modulate ongoing levels of aggressive behaviour. Consistent with the results of this pharmacological study, 39, $X^{Y*}O$ male mice, which have low levels of DHEA (and presumably elevated levels of DHEAS), are hyper-aggressive towards their cagemates[77].

To date, there is little evidence from human studies that suggests an equivalent role for STS in modulating aggression: to our knowledge, abnormally high levels of overt aggression have not been reported in cases of *STS* deletion, and within our Cardiff ADHD sample we did not detect association between a number of polymorphisms within *STS* and DSM-IV aggressive symptoms (albeit the case that the sample displayed low overall levels of aggression)[3]. There are two possible reasons for this apparent cross-species discrepancy: first, mouse brain function may be differentially affected by changes in STS levels, or the social structures imposed within groups of laboratory-housed mice might be conducive to eliciting aggression. Alternatively, the phenotype of elevated aggression may be present in *STS*-deficient humans but it may be more subtle than in mice, or only observable upon provocation.

Whilst there is fairly convincing cross-species evidence indicating a role for STS in attentional processes, and some data consistent with a role for the enzyme in aggression (in rodents at least), data suggesting a modulatory role in other behavioural phenotypes is more limited; this preliminary evidence is summarised below.

Impulsivity

In addition to being inattentive relative to 40, XY control mice, *STS*-deficient 39, $X^{Y*}O$ mice appear to be less impulsive, as indexed by their tendency to make fewer premature responses to a stimulus in the 5-CSRTT[71], and to better withhold responding on a murine analogue of the human Stop Signal Reaction Time Task (SSRT); pharmacological manipulation of the STS axis (*i.e.,* COUMATE and DHEAS administration) also enhanced behavioural inhibition on the SSRT. These data hint that STS deficiency in humans may also confer reduced impulsivity. Most research in psychiatry has focussed on pathological hyper-impulsiveness rather than the consequences of hypo-impulsivity, so exactly how this might be manifest is unclear - perhaps such individuals would show a tendency towards apathy or extreme risk aversion? To date, there is no information available regarding whether XLI patients are particularly apathetic or cautious. At the neuropsychological level, we might predict that *STS*-deficient subjects, like 39, $X^{Y*}O$ mice, would demonstrate enhanced "stopping" on the SSRT. Testing for association between polymorphisms across the *STS* gene and DSM-IV impulsive symptoms in a small sample of boys with ADHD revealed no significant findings[3]. However, such a result is perhaps not surprising if *STS* variation does generally result in reduced impulsivity, given that the ADHD population is, by definition, ascertained on the basis of abnormally high levels of impulsivity.

Psychotic and mood disorders

Postpartum psychosis (PP) is a severe psychiatric condition occurring shortly after birth in 1-2 of every 1000 new mothers; it is characterised by hallucinations/delusions, cognitive disorganisation, mood changes and

sleep abnormalities and can occasionally result in self- or infant-directed harm[78]. Whilst biology undoubtedly plays a role in PP susceptibility, as yet, well-defined risk factors are few and far between. The biggest risk factor appears to be a personal or family history for psychiatric (psychotic) illness, with a strong and consistent relationship between prior bipolar disorder diagnosis and vulnerability to PP; other risk factors include the extent to which circulating maternal oestrogen levels plummet following expulsion of the placenta, and levels of maternal stress[78]. It has been proposed that maternal STS deficiency might influence PP risk on the basis of several observations (described in detail in a recent paper[79]): (1) levels of the STS enzyme in the mammalian maternal brain specifically increase following parturition, hence perturbation of the STS axis as a consequence of enzyme deficiency may particularly impact upon behaviour at this timepoint; (2) the enzyme is highly expressed in the placenta where it is involved in the biosynthesis of oestrogen precursors; decreased levels of circulating oestrogens during pregnancy and in the postpartum period as a consequence of Sts deficiency may predispose to psychosis; (3) *STS* is a candidate gene underlying a quantitative trait locus for postpartum behavioural disturbance in pigs; (4) as discussed above, *STS*-deficiency in mice and humans predisposes to inattention (or "cognitive disorganisation") and occasional aggression; and (5) *STS* is highly expressed in the thyroid gland, an organ whose dysfunction is often noted in cases of PP. Given the overlap between bipolar disorder and PP, it is interesting to note that in a recent meta-analysis of gene expression data from *post mortem* brain samples from patients with bipolar disorder, *STS* was one of just a handful of genes whose expression was consistently downregulated[80]; thus it plausible that *STS* deficiency predisposes primarily to bipolar disorder and thereafter to PP, or to PP directly, or to both disorders *via* shared neurobiological mechanisms. It is also noteworthy that the estimated prevalence of female heterozygosity for a deleterious *STS* mutation (based on prenatal screening and XLI rates) is comparable to that of PP (*i.e.,* about 1 in 750-3000 females), that the neurosteroid system has previously been implicated in psychotic disorders and in other postpartum conditions[81], that two female cases of paranoid schizophrenia possessed cytogenetic deletions spanning *STS*[61], and that deficiency for the *STS* paralogue, *ARSA* (arylsulfatase A), has been linked to psychotic phenotypes and postpartum depression[82-84].

A relationship between STS deficiency and a second mood disorder, unipolar depression, might also be considered, although currently the evidence for such a link is weak or anecdotal. One of two females completely deficient for enzyme activity according to biochemical measurements (and therefore homozygous for an undefined *STS* mutation) was reported to have a history of depression[85]. Moreover, we are aware of one United Kingdom family in which X-linked ichthyosis appears to co-segregate with debilitating depression/anxiety.

Other behavioural endophenotypes

39, $X^{Y*}O$ mice are hyperactive, particularly within a novel environment and during the night (*i.e.,* during their active phase)[77], and there is a significant inverse relationship between evening activity in a novel environment and systemic levels of the STS product DHEA[86]. 39, $X^{Y*}O$ mice also exhibit increased response rates within operant behavioural tests where only one type of response is required, which may reflect a greater tendency towards perseverative responding[86,87]. Finally, these mice tend to show heightened emotional reactivity relative to wildtype controls as indexed by their lack of willingness to enter aversive (open, brightly-lit) spaces, and their increased levels of urination and defecation in such spaces[77]. As 39, $X^{Y*}O$ mice also lack the PAR gene *Asmt*[86], theoretically, the hyperactivity, perseverative and emotional reactivity phenotypes in 39, $X^{Y*}O$ mice could be due to the loss of *STS* and/or *Asmt* (particularly given that the former and latter phenotypes are not elicited upon acute COUMATE administration[77]). However, the inverse association between DHEA levels and hyperactivity tends to argue for a specific role for STS in this behaviour. To date, it has not proved possible to generate single-gene *STS* and *Asmt* knockout rodents; should such animals be created in the future, they could be used to dissociate between behavioural effects arising dues to STS and/or ASMT deficiency.

Hyperactivity and behavioural inflexibility (perseveration) have not consistently been reported as (endo) phenotypes associated with cases of STS deficiency in humans. This could be because these phenotypes in mice are largely due to ASMT deficiency, because there is no association, because the phenotypes are relatively minor and do not impair everyday function, or because no systematic, objective, case-controlled behavioural studies have yet been performed for XLI (*e.g.,* using activity monitors). The observation that 39, $X^{Y*}O$ mice exhibit more anxiety-related behaviours than their wildtype counterparts is consistent with anecdotal evidence suggesting anxiety in XLI patients, but clearly the veracity and magnitude of this association requires further exploration.

EFFECTS ON NEUROANATOMY AND NEUROCHEMISTRY: INSIGHTS FROM ANIMAL MODELS

The comparative rarity of XLI patients, together with the relative inaccessibility of the human brain, means that, to date, little is known about the neuroanatomical and neurochemical sequelae of STS deficiency in man. Individuals in which *STS* is deleted have been reported to exhibit cortical malformations including polymicrogyria[88] and heterotopia[89,90]; whilst these manifestations may be consistent with a role for STS in the developing cortex[3], given that these individuals lack multiple genes at Xp22.3, these abnormalities might equally likely be a consequence

of the absence of function of one or more contiguous brain-expressed genes (*e.g., HDHD1A*). To uniquely ascribe these cortical phenotypes to STS deficiency, it will be necessary to examine the brains of individuals with nonsense point mutations within *STS* either by *in vivo* neuroimaging or through analysing *post mortem* tissue.

Rodent models are far more amenable to neurobiological investigation than humans, and ongoing studies in genetic and pharmacological models have provided interesting initial clues as to the neurochemical mechanisms underlying STS deficiency effects on behaviour. Relatively crude analyses of whole tissue monoamine levels in the 39, $X^{Y*}O$ mouse have identified brain region-specific changes in the serotonin (5-HT) system (notably elevated 5-HT levels in the striatum and hippocampus)[86,87]; these mutant mice also have increased hippocampal expression of the *Htr2c* gene (encoding the 5-HT_{2c} receptor) and reduced striatal levels of the noradrenaline metabolite 4-hydroxy-3-methoxyphenylglycol[87]. Correlational analyses have indicated a positive linear relationship between hippocampal 5-HT levels and response rate/behavioural perseveration across two independent behavioural paradigms[86,87] and between striatal 5-HT levels and activity[87]; whilst these observations suggest that the 5-HT system abnormalities may affect these behavioural endophenotypes, an explicit causal link between the variables has yet to be established. Interestingly, serotonergic system dysfunction (including disruption of 5-HT_{2c} receptor expression/function) has been implicated in many of the psychiatric phenotypes linked to STS deficiency including inattention[91,92], aggression[93], impulsivity[94], PP[95], anxiety and depression[96,97]. More refined analyses of the relationship between analogues of these behavioural/psychiatric outcomes and 5-HT perturbation in the 39, $X^{Y*}O$ mouse will be useful, notably investigating whether neurochemical changes within specific sub-regions of the hippocampus and striatum underlie the behavioural abnormalities. Although the most parsimonious explanation for the neurochemical findings is STS deficiency[86], it is formally possible that they could arise due as a consequence of *Asmt* gene deletion. Again, examining single gene knockouts and/or assaying whether 39, $X^{Y*}O$ phenotypes are recapitulated by acute enzyme inhibition will aid in distinguishing between these scenarios. Should a discrete STS-dependent change in 5-HT function be confirmed in mice, its potential functional relevance to human phenotypes might be tested through using positron emission tomography with serotonergic ligands in XLI patients[98].

To date, the neuroanatomy of the 39, $X^{Y*}O$ mouse has not been examined; future analyses might initially look for gross abnormalities in hippocampal and striatal morphology for example, before investigating more subtle changes in cell number or subtype in these regions. Given the suggestion above that XLI may be associated with cortical abnormalities, examining the development and structure of the cortex in *STS*-deficient mice may also be warranted.

Rat studies in which STS is inhibited have also begun to shed some light on the neurochemical pathways influenced by enzyme dysfunction. Again, these have emphasised the hippocampus, a key site of neurosteroid-mediated neurogenesis[99], as an important locus of ongoing Sts action. Initial studies using estrone-3-O-sulfamate (EMATE) as an inhibitor indicated that inducing acute enzyme dysfunction, particularly in combination with substrate (DHEAS) administration, could benefit learning and/or memory formation[100]. However, EMATE is oestrogenic[101], and thus it is difficult to ascertain whether its effects on cognition are due to its inhibitory and/or its oestrogenic role. In later studies, systemic enzyme inhibition using the compound [p-O-sulfamoyl-N-tetradecanoyl tyramine (DU-14), a compound with lower oestrogenicity than EMATE] administered chronically was shown to increase brain levels of DHEAS[102], enhance hippocampal release of the neurotransmitter acetylcholine[103], and result in improved learning, spatial memory and context-dependent fear memory[104,105]. The acetylcholine system has a long association with attentional function, and one obvious route through which STS axis variation might influence attention is *via* this intermediary mechanism[106]. Thus, future work might examine the effects of STS inhibition, or *STS* gene deletion, on acetylcholinergic function in the hippocampus and other brain regions implicated in attention, and how these induced neurochemical changes might then relate to measures of (in) attention. Within the hippocampus, various serotonergic receptors are involved in controlling acetylcholine release[107]; hence, it will also be interesting to see whether there is any relationship between serotonergic and acetylcholinergic abnormalities induced as a consequence of STS deficiency in this structure.

CONCLUSIONS AND FUTURE DIRECTIONS

In the preceding text we have marshalled evidence from a variety of sources which indicates that STS deficiency may elicit a multitude of brain and behavioural phenotypes of relevance to psychiatric vulnerability. Clearly there is a need for further systematic work to specify the precise neural, behavioural and psychiatric endophenotypes arising from this molecular abnormality, and to investigate their prevalence; such work may provide more general insights into behavioural and cognitive processes that commonly go awry in psychiatric conditions (*e.g.,* attention). Ideally, this work would involve the examination of individuals with discrete mis-/nonsense point mutations within the *STS* gene, or deletions solely encompassing *STS*, in whom any phenotype could not be ascribed to missing contiguous genes; however, given the low frequency of such mutations within the population, identifying such individuals will be difficult, and establishing reliable prevalence figures for particular phenotypes will be challenging. In light of the arguments outlined above,

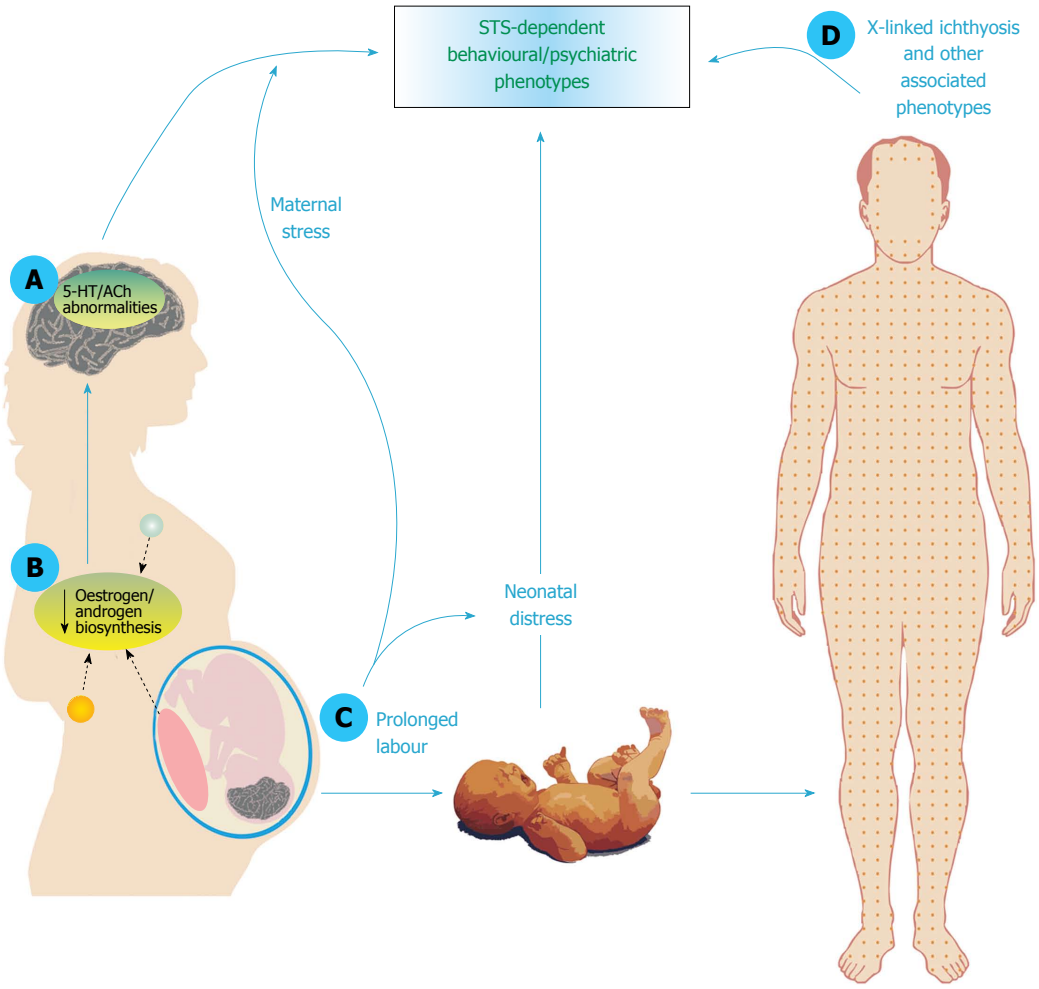

Figure 1 Mechanisms through which steroid sulfatase deficiency could theoretically influence neurobehavioural and psychiatric phenotypes. A: Loss of activity in the brain could directly influence neurodevelopment or ongoing function (*e.g., via* effects on the serotonergic or acetylcholinergic systems); B: Loss of activity in steroidogenic tissues (*e.g.,* placenta, ovaries, mamary gland) could affect neurodevelopment and brain function *via* effects on levels of circulating oestrogens and androgens; C: High levels of stress associated with prolonged labour (as a consequence of enzyme dysfunction in the fetal portion of the placenta) could both potentiate the development of postpartum illness in the mother, and could adversely affect neurodevelopment in the offspring; D: Living with potentially stigmatising conditions such as ichthyosis and male pattern baldness could feasibly increase the risk of developing disorders such as depression and anxiety. STS: Steroid sulfatase.

we propose that it would be worthwhile for patients with confirmed/suspected XLI to be asked by clinicians about any behavioural problems that they might have experienced, given that any link between dermatologic abnormalities and such difficulties may not be intuitive.

Should elevated rates of psychiatric illness be identified in enzyme-deficient individuals, it will be important to characterise their mechanistic basis *i.e.,* whether they are related to loss-of-function of the enzyme in the brain *per se*, to the pleiotropic, non-brain effects of protein dysfunction, or to both (Figure 1). STS deficiency could influence brain function and behaviour directly (*e.g.,* through modulating the serotonergic or acetylcholinergic systems). Alternatively, lack of enzyme expression in steroidogenic organs such as the placenta, gonads and mammary gland[108] could result in reduced levels of circulating steroid hormones (notably androgens and oestrogens) and subsequent downstream organisational and/or activational effects on brain development and function. Animals models in which STS function is perturbed will be of use in distinguishing between these two possibilities. Furthermore, it is conceivable that an increased risk of some psychiatric disorders (*e.g.,* depression and anxiety) could result from individuals having to live with potentially disfiguring somatic conditions associated with enzyme deficiency including ichthyosis[42], hypogenitalism[109], or male-pattern baldness[110]; in support of this notion, one study has shown that patients with ichthyosis have a lower health-related quality of life[111]. Finally, it is conceivable that STS deficiency in the offspring could both result in increased risk of psychiatric illness in that individual simply as a consequence of neonatal distress arising from prolonged maternal labour[112-114], and, by the same general stress-inducing mechanism, could increase risk of postpartum mental illness in the mother[115]. To determine whether these latter mechanisms may be important, comparison of rates of behavioural/psychiatric abnormalities in STS-deficient cases with those in subjects with other similar skin conditions for example, or exposed to other causes of prolonged labour, may be valuable.

An alternative strategy for determining the consequences of acutely impaired STS function may be to explicitly test for brain and behavioural alterations in subjects administered enzyme inhibitors. 667-COUMATE (Irosustat) has been proposed as a treatment for hormone-dependent cancers[116,117] and for endometriosis[118], conditions where biosynthesis of oestrogens and androgens must be restricted. Although no obvious psychological side-effects of 667-COUMATE treatment were reported in the first clinical trial of the drug in breast cancer patients[119], the animal data discussed above suggest that subtle effects on cognition (attention, impulsivity) and behaviour (aggression) might be anticipated. In assessing whether this is the case, potential confounding variables such as baseline rates of depression, age, or the potential behavioural effects of co-administered therapeutic drugs, in patients would have to be considered.

Finally, taking a conceptually different approach, it will be interesting to see whether subjects with cytogenetic duplications encompassing *STS* exhibit any clear brain or behavioural phenotypes. However, in most, if not all, of these cases, any data will be confounded by duplication of contiguous brain-expressed genes. To date, the small number of cases with Xp22.3 duplications reported in the literature either do not appear to exhibit any severe neuropsychological phenotypes[120], or present with relatively non-specific phenotypes such as learning disability and/or developmental delay[121] depending upon the size of the duplication.

Understanding if, and how, STS deficiency influences vulnerability to psychiatric illness will be important in terms of counselling for XLI (and potentially TS), and additionally may highlight novel therapeutically-amenable targets for common aspects of psychological dysfunction.

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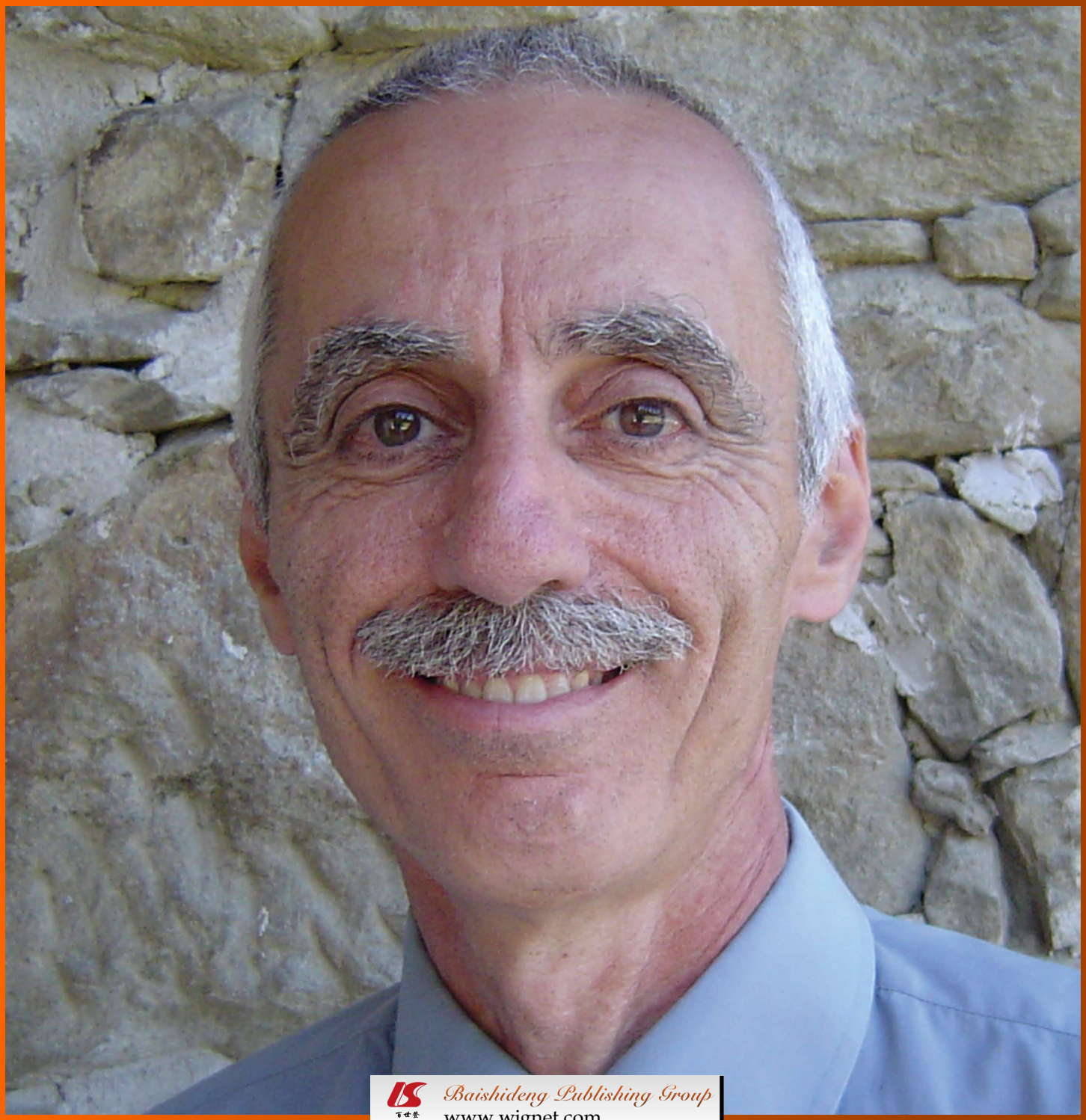

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BRIEF ARTICLE

High-potency sucralfate prevents and rapidly reverses chemo-radiation mucositis in a patient with stage 4b head and neck cancer

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Abstract

AIM: To study usefulness of high-potency sucralfate (HPS) in a patient with chemoradiation mucositis and discuss its mechanism of action.

METHODS: HPS, a non-covalently cross-link of sucralfate, cations and bidentate anionic chelators, has a maintains a surface concentration of sucralfate 3 h following administration that is 7-23 fold that possible with standard-potency sucralfate. The accelerated mucosal healing and pain alleviation of HPS in patients with erosive esophageal reflux, prompted its use in this patient with chemoradiation mucositis of the oropharynx and alimentary tract. A literature-based review of the immuno-modulatory effects of sucralfate is discussed.

RESULTS: Within 48 h of intervention: (1) there was complete disappearance of oral mucositis lesions; tenderness with (2) patient-reported disappearance of pain, nausea and diarrhea; patient required (3) no opiate analgesia and (4) no tube-feeding supplements to regular diet. Dysgeusia and xerostomia persisted. A modified Naranjo Questionnaire score of 10 supported the likelihood that HPS intervention caused the observed clinical effects. No adverse reactions noted.

CONCLUSION: In this patient HPS was useful to treat chemo-radiation mucositis of the oropharynx and alimentary tract. HPS may directly or indirectly facilitate an immunomodulatory mechanism involving accelerated growth factor activation, which may be a new target for therapeutic intervention in such patients.

Key words: Sucralfate; Mucositis; Chemoradiation; Immuno-modulation; Cytokines; Intra-epithelial lymphocytes; Growth factors

Core tip: Mucositis is a debilitating and costly consequence of chemo-radiation. Most mucositis treatments are palliative. Conversely, high-potency sucralfate (HPS) may be definitive. Patients with stage 4b head neck cancer, at high risk for developing mucositis, require gastrostomy tubes as an alternative to oral feeding. The use of HPS in this cancer patient prevented mucositis, allowing continuance of standard oral diet. Midway through chemo-radiation, though noncompliant discontinuation of HPS, by patient led to the emergence oral and alimentary mucositis, 2 d following resumption

of HPS, mucositis disappeared, a normal oral diet was maintained and no analgesia was required.

McCullough RW. High-potency sucralfate prevents and rapidly reverses chemo-radiation mucositis in a patient with stage 4b head and neck cancer. *World J Transl Med* 2013; 2(2): 13-21 Available from: URL: http://www.wjgnet.com/2220-6132/full/v2/i2/13.htm DOI: http://dx.doi.org/10.5528/wjtm.v2.i2.13

INTRODUCTION

Healing of mucositis erosions induced by chemo-radiation in cancer patients involves a balanced interplay between cytokines (pro- and anti-inflammatory), chemokines and growth factors[1]. Transforming growth factor β (TGFβ), which is upregulated by epithelial growth factor (EGF), TGFα, pro-inflammatory interleukin-1β and interferon γ[2] appears to be key in the pathobiology of oral mucositis, and likely in alimentary mucositis as well. Standard potency sucralfate avidly aid in growth factors[3] activation but has no significant clinical affect on chemo-radiation induced mucositis.

Signs and symptoms of oral mucositis (its associated symptoms and physical findings) have been standardized with most clinicians using the World Health Organization (WHO) grade classification system in Table 1[4]. The severity of mucositis-related alimentary toxicity, have two main grading scales one shown in Table 2, by the European Organization for Research and Treatment of Cancer/Radiation Therapy Oncology Group[5] and the other by the WHO[4]. In patients with advanced grades of mucositis (Grade 2 and 3), dose reduction is required in 60% of them, and 30% require discontinuation of chemotherapy regimens[6,7].

Adequate nutritional support is also a major problem. Regardless of cancer type or dose of treatment, 70% of patients with Grade 3 or 4 mucositis, require tube-feeding to supplement caloric and hydration needs. In patients undergoing hematopoietic stem cell transplant (HSCT), nearly 87% require tube-feeding with 80% requiring narcotic analgesics. There are economic issues related to mucositis as it increases the cost of care. Patients with solid tumors receiving chemotherapy who develop oral mucositis are hospitalized 4.3 d longer at a cost increase of 6277 per cycle[8]. Bone marrow transplant patients with oral mucositis require additional days of hospitalization resulting on average in increased hospital charges of 42749 per patient[9].

Episodes of mucositis are predictable. It is the most significant side effect of patients with head and neck cancer[10,11] receiving high-dose chemotherapy or radiation therapy. Incidence of severe oral mucositis approaches 100% in patients with stage 3 or 4b head and neck cancer receiving high dose radiation. Nearly 75% of patients undergoing HSCT experience advanced grades of both oral and gastrointestinal (GI) mucositis, particularly if methotrexate is used to prevent graft-*vs*-host disease[6]. High rates of alimentary mucositis, upwards of 20%-50%, occur with the use of 5-fluorouracil, capecitabine or tegafur to treat tumor and metastatic sites[6,12,13]. Similarly, 20%-60% of patients receiving chemotherapeutic antimetabolites such as methotrexate develop dose-dependent alimentary mucositis per cycle[6,12].

Clearly effective management of oral and alimentary mucositis would address patients' pain, rate of infection, nutritional states as well as recurrent hospitalizations, costs of care and optimization of treatment dose. Most FDA cleared interventions garner only a "standard of clinical practice" justification for their use and await expanded evidence-based examination[14]. Few cancer support therapies qualify for advanced guideline status, as the level of clinical efficacy fall short of that established by the Multinational Association of Supportive Care in Cancer (MASCC)[15,16].

The most recent guidelines on the treatment of oral mucositis include use of antimicrobial lozenges, benzydamine, oral cryotherapy, keratinocyte growth factor-1, and low-level laser therapy. To treat alimentary mucositis, MASCC panel recommends amifostine, ranitidine or omeprazole for upper GI mucositis and sulfasalazine 500 mg twice daily, sucralfate enemas, loperamide or octreotide 100 mg subcutaneously twice daily for lower GI mucositis[14].

No single agent satisfactorily addresses the occurrence of mucositis throughout the length of GI tract. Specifically, the 2005 MASCC guidelines recommended against the use of sucralfate for the prevention or treatment of radiation induced oral mucositis.

However, the patient in this report with oral and alimentary mucositis responded to high-potency sucralfate (HPS). HPS is original potency sucralfate with enhanced muco-adherence achieving high mucosal surface concentration.

Its presumed mechanism of action to be discussed later may involve engagement of nascent growth factors and neutralizing the polarity of ion-gated mucosal nociceptors.

MATERIALS AND METHODS

This was an interventional study in a patient with advanced stage 4 head and neck cancer undergoing concurrent chemoradiation and thus prone to develop severe oral and alimentary mucositis. The setting of the study was an outpatient department of medical oncology, radiation medicine and internal medicine. The patient provided informed consent and was enrolled in a compassionate use program sponsored by Mueller Medical International who provided ProThelial™ a proprietary formulation of HPS.

HPS

HPS has been shown to mitigate nausea, vomiting and diarrhea as well as accelerates healing of GI erosions in

Table 1 Grade scales for the assessment of oral mucositis

World Health Organization Grade	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Function	Painless ulcers, erythema or mild soreness	Painful erythema, edema, or ulcers but can eat solids	Painful erythema, edema, or ulcers and cannot eat solids	Alimentation is not possible; dependence on Ⅳ and feeding-tube	
Clinical Exam	Erythema of the mucosa	Patchy ulcerations or pseudomembranes	Confluent ulcerations or pseudomembranes; bleeding with minor trauma	Tissue necrosis; significant spontaneous bleeding; life-threatening consequences	Death
Symptoms	Minimal symptoms, normal diet; minimal respiratory symptoms but not interfering with function	Symptomatic but able to eat and swallow modified diet; respiratory symptoms interfering with function but not with activities of daily living	Symptomatic and unable to adequately aliment or hydrate orally; respiratory symptoms interfering with activities of daily living	Symptoms associated with life-threatening consequences	Death

Table 2 European Organization for Research and Treatment of Cancer/Radiation Therapy Oncology Group and the World Health Organization toxicity criteria acute chemoradiation morbidity

	Scale for gastrointestinal toxicity				
	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Esophagus toxicity grade	None	Mild fibrosis; Slight difficulty in swallowing solids; No pain on swallowing	Unable to take solid food normally; Swallowing semi-solid food; Dilation may be indicated	Severe fibrosis; Able to swallow only liquids; May have pain on swallowing; Dilation required	Necrosis/Perforation Fistula
Small bowel toxicity grade	None	Mild diarrhea; Mild cramping; Bowel movement 5 times daily	Moderate diarrhea and colic; Bowel movement > 5 times daily	Obstruction or bleeding, requiring surgery	Necrosis/Perforation Fistula
Colorectal toxicity grade	None	Increased frequency or change in quality of bowel habits not requiring medication, rectal discomfort not requiring analgesics; Slight rectal discharge or bleeding	Diarrhea requiring parasympatholytic drugs, mucous discharge not necessitating sanitary pads, rectal or abdominal pain requiring analgesics; Excessive rectal mucus or intermittent bleeding	Diarrhea requiring parenteral support, severe mucous or bloody discharge necessitating sanitary pads/abdominal distension (flat plate radiograph demonstrates distended bowel loops)	Acute or subacute obstruction, fistula or perforation; gastrointestinal bleeding requiring transfusion; abdominal pain or tenesmus requiring tube decompression or bowel diversion
World Health Organization colorectal Toxicity grade	None	Increase of 2-3 stools per day over pretreatment	Increase of 4-6 stools per day, or nocturnal stools, or moderate cramping	Increase of 7-9 stools per day, or incontinence, or severe cramping	Increase of > 10 stools per day or grossly bloody diarrhea, or need for parenteral support

man and animals. It is prepared by suspending regular-potency sucralfate in a select solution of cations and bidentate anionic chelators[17]. In this patient, doses of HPS suspension containing 1.5 g of sucralfate were self-administered three times daily for 2 d at the onset of mucositis symptoms and signs. Then twice daily dosing was continued throughout treatment course up to 2 wk following cancer therapy.

Outcome measures

There were two primary outcome measures and two secondary outcome measures. Primary measures consisted of the limitation or disappearance of visible oropharyngeal lesions and patient reported alimentary symptoms of pain, nausea, vomiting and diarrhea. Secondary outcome measures comprised of any need for opiate analgesia and tube-feeding supplementation of oral diet, and the score on a modified Naranjo Questionnaire[18]. The latter was employed to assess the probability of the intervention causing the observed clinical effects.

Case presentation

The patient was a 43-year-old male home health aide, divorced, with four children, who seldom drank alcohol and had stopped smoking 2 years prior to presentation to otolaryngologist but had a 17 pack year history of smoking. His mother died of metastatic breast cancer at age 52 years and his father died of unknown cause.

He presented with a 1 year history of swelling in his right neck for which he had received multiple courses of antibiotics with no appreciable change. With progressive swelling he had developed a 6-mo history of fullness in the back of his throat, a sensation occasionally associated with gagging during meals. He was referred to an otolaryngologist for evaluation of neck swelling and worsening gag.

On physical exam he was 73 inches tall, weighed 235 lbs and had an 8 cm × 6 cm neck mass below the right mandible extending to the angle of the jaw. Direct fiber-optic examination of the throat revealed a large mass at the base of the tongue. The biopsy of the right neck

Table 3 Modified naranjo probability of intervention-caused response

	Questions	Yes	No	Don't know	Case report
1	Are there previous conclusive reports on this response?	1	0	0	0 don't know
2	Did the response appear after the intervention was administered?	2	-1	0	+2 Yes
3	Did the response disappear when the intervention was discontinued?	+1	0	0	+1 Yes
4	Did the response reappear when the intervention was re-administered?	+2	-1	0	+2 Yes
5	Are there alternative causes that could on their own have caused the reaction?	-1	+2	0	+2 No
6	Did the reaction reappear when a placebo was given?	-1	+1	0	+1 No
7	Was the intervention detected in the blood in concentrations known to be toxic?	+1	0	0	0 No
8	Was the response more apparent when the dose was increased, or less apparent when the dose was decreased?	+1	0	0	0 No
9	Did the patient have a similar response to the same or similar intervention in any previous exposure?	+1	0	0	+1 Yes
10	Was the response confirmed by any objective evidence?	+1	0	0	+1 Yes
Patients total score					10

mass revealed a poorly differentiated squamous cell carcinoma. A computed tomography (CT) scan of the neck revealed massive right internal jugular lymphadenopathy from the angle of the jaw to the level of the thyroid measuring 6 cm × 4 cm in cross section. There was a 2-cm mass in the right tonsillar area at the base of the tongue which represented the primary tumor. A CT of the brain and chest was negative for metastatic disease.

Formal cancer diagnosis for this patient was squamous cell carcinoma of the base of the tongue classified as a T3N3M0 stage 4b. His case was presented to the hospital tumor board and it was recommended that he undergo a concurrent course of chemoradiation with a modified radical neck dissection.

Clinical course

Per institution protocol for all stage 4b head and neck cancer expected to develop oral and alimentary mucositis, the patient underwent placement of percutaneous G-tube and given a home supply of tube feeding solution. His concurrent chemoradiation consisted of weekly transfusion of Paclitaxel and Carboplatin with radiation totaling 71 Gy for base of the tongue, 71 Gy to the tumor mass itself and an additional radiation dose of 59 Gy to right sided regional nodes.

By week 2 patient develop WHO Grade 2 oral mucositis, and WHO Grade 1-2 esophageal and small bowel mucositis with painful swallowing, nausea, occasional vomiting and frequent loose stools. Patient was prescribed Prothelial™, a potency-enhanced sucralfate suspension 1.5 g, 3 times daily for 2 d, and then a maintenance dose of 1.5 g twice daily.

RESULTS

Primary outcome measures for HPS

All primary outcome measures were met. There was visible resolution of mucosal erosions and patient reported absence of nausea and diarrhea within 48 h. In week 4 feeling well and assuming that he no longer needed it, the patient stopped HPS for 10 d against protocol while under chemoradiation. He suffered a recurrence of oral lesions, nausea, and diarrhea. Two days following resumption of HPS suspension patient's recurrent symptoms and ulcers had cleared.

Secondary outcome measures for HPS

All secondary outcome measures were met. Patient required no opiate or non-opiate analgesia while on HPS suspension. Additionally while on HPS, the patient did not require use of the feeding tube nor of caloric supplementation as he was able to continue pre-treatment diet, tolerating both solid food and liquids. At the start of chemo-radiation, the patient was 35 lbs overweight for his height of 73 inches, weighing 235 lbs. While on HPS, he maintained his ideal body weight of 198 lbs.

Naranjo Algorithm for HPS

The Naranjo Algorithm is a validated questionnaire designed to determine the likelihood that an observed clinical effect in a patient exposed to a drug can be attributed to the drug or other factors[18]. Though generally used to investigate adverse drug reactions, the Naranjo Questionnaire, in its most basic sense, is a validated method to assess whether a drug or intervention can be linked to a subsequent clinical reaction (or response). Thus it was reasonable to use the algorithm to assess the likelihood that the observed but unexpected clinical response in this patient was due to HPS.

Most patients with stage 4b head and neck cancer treated with radiation, Paclitaxel and Carboplatin concurrently develop oral and alimentary mucositis due to required concurrent chemo-radiation[10,11,19] and indeed by week 2 this patient developed symptomatic oral and alimentary mucositis of the GI tract. Relevant to Naranjo Algorithm is that patient symptoms and signs disappear within 2 d of introduction of HPS, recurred when the patient stopped the sucralfate intervention for 10 d, but then disappeared 2 d following the resumption of HPS.

Table 3 shows the Naranjo score of 10 in this patient treated with HPS. Ordinarily, a score > 9 implies a definite drug-effect association, a score between 5-8 implies

probable association, a score between 1-4 implies a possible association while a score of "0" implies doubtful association. The Naranjo score of 10 for HPS implies that there was likely a "definite" probability that the intervention was associated with the observed clinical improvement in this patient.

DISCUSSION

Rubenstein *et al*[15] reviewed 38 agents and modalities prescribed by physicians from 1985 through 2004 for the management of both oral and alimentary mucositis. By mechanism of action these agents are grouped as anti-inflammatories, anti-infectives, anti-oxidants, immuno-modulators, muco-adhesives, cytoprotectants, anti-ulcerants and biophysical interventions. HPS is a muco-adhesive cytoprotectant that appears to facilitates immuno-modulatory prevention and reversal of mucositis through out the GI tract.

MASCC guidelines[15] do not recommend the use of standard potency sucralfate to treat or prevent mucositis. However, in the patient of this report, HPS prevented oral and alimentary mucositis. When HPS was inadvertently discontinued, when both oral and alimentary mucositis recurred, due to patient's inadvertent noncompliance, HPS treated it fairly rapidly within 48 h.

For this patient the use of HPS obviated the need to reduce or in any way alter an aggressive treatment regimen for the cancer. There was no use of opiate analgesia. Tube-feed supplementation was unnecessary as well. Obviously, an expanded evaluation of HPS is required as these responses were observed in a single patient.

Translational medicine view

Mucositis is a long-standing unmet medical need in supportive care of cancer treatment. A positive clinical effect of HPS on oral and alimentary mucositis stands in stark contrast to the exclusion of sucralfate from MASCC guidelines - guidelines that greatly impacts medical practice and research. Indeed, expanded clinical trials are necessary to ascertain the permanence (if any) of this HPS observation. Nevertheless the question remains as to mechanism whereby HPS could possibly ameliorate signs and symptoms of mucositis. The following mechanism proposed in this report utilized methods of translational medicine to integrate basic science input from multi-disciplines of study. This mechanism of action for HPS centers on the efficient activation of mucosal growth factors near the site of mucosal injury or assault. From the viewpoint of translational medicine, efficient activation of nascent mucosal growth factors is a therapeutic target for others in the field, focusing efforts on the discovery of other, potentially better, agents to treat and prevent mucositis. The remainder of this report is devoted to a fundamental standard of translational medicine - understand the actions of an intervention so as to use its principle to unearth additional potentially better interventions.

Understanding sucralfate: Its potency and multi-modal mechanism of action

Sucralfate is a polyanionic disaccharide that exerts the totality of its clinical effects through physical contact with the mucosa. It is non-systemic. The classic understanding of sucralfate's mode of action is that it acts as a "bandage", as a physical barrier covering the mucosal, supplemented by chemo-adsorption actions of sucralfate against pepsin and bile salts[20].

However, Hollander *et al*[21] reported other near-immediate mucosal effects following administration of sucralfate. Within 10 min of contact on the mucosa and at appropriate doses, sucralfate initiates epithelial regeneration and stimulates (1) secretion of a mucus gel; (2) the release of bicarbonate beneath this gel; and (3) the secretion of somastatin and prostaglandin E. Unknown at the time of their report, these effects were mediated by direct engagement of focal growth factors by sucralfate. "In appropriate doses" is the operative phrase. The effects of sucralfate reported by Hollander *et al*[21] occurred at doses five to twenty times the allowable human dose of 14 mg/kg. Rats received single doses of 70-280 mg/kg. In man, the latter high oral doses can result in bezoar formation in man.

Potency enhancement of sucralfate

Potency of sucralfate is defined as the extent of clinical effect associated with surface concentration of sucralfate achieved following administration. Standard potency sucralfate cannot treat or prevent oral or alimentary mucositis. However, the potency of sucralfate can be greatly enhanced by suspending standard potency sucralfate in a solution of multivalent cations buffered by multi-dentate anionic chelators. The resultant "cross-linked" sucralfate is believed to facilitate orderly layering of sucralfate on the mucosa and upon itself. Orderly layering on the mucosa and upon itself could account for the multifold elevation of surface concentration of sucralfate in HPS per dose without a commensurate increase in its formulary strength in grams per milliliter. Three hours following administration, HPS maintains mucosal concentrations of sucralfate at least 7 fold that expected for standard potency sucralfate of equal formulary strength[17]. On ulcerated or irritated enteric lining the mucosal concentrations of sucralfate from HPS is 23 fold above that expected for standard potency sucralfate of equal formulary strength.

These multiples of surface concentration achieved by HPS are equivalent to the augmented doses of sucralfate used unsuspectingly by Hollander *et al*[21]. This potency enhancement effect is retained when HPS suspension is dehydrated and administered as a powder in a capsule.

Immuno-modulatory and depolorization mechanism of action

The exact mechanism of action of HPS is unknown. However, relying on the literature across several disciplines of biomedical sciences, a case can be made that HPS (as well as other polyanionic compounds) provides two significant effects on contact: firstly an immuno-

modulatory effect through non-specific but high-affinity interactions with growth factors and secondly, an ionic depolarization of activated ("firing") ion-gated nociceptors embedded in the mucosa. Ion-gated nociceptors embedded within the mucosa give rise to pain, nausea and vomiting. Polyanionic stabilization of ion-fluxes in activated nociceptors reduce their firing, and thereby the sensation of pain, nausea and vomiting on contact. Activated growth factors of the GI tract maintain normal mucosal function and epithelial integrity. The following outlines salient features of GI function that are most likely influenced by the topical application of HPS.

Mucosal physiology of GI tract

The mucosal lining of the GI tract is tasked with both digestive and defensive functions[22,23]. For the purposes of defense, the GI lining has an embedded array of specialized mucosal receptors (nociceptors)[23,24], intramucosal (epithelial) lymphocytes[25-34] sub-mucosal immune cells[35-42] and sub-mucosal sensory and effector neurons[23,24]. Mucosal nociceptors are gated-ion type receptors that register acidity, pressure, stretch and pain and are innervated by A-fiber and C-fiber neurons[23-25].

Specialized mucosal lymphocytes known as intra-epithelial lymphocytes (IELs) are responsible for surveillance and detection of unwanted agents, toxins and substances[23-25]. There are three major subpopulations of such cells[26,31,32]. Two major subpopulations of α-β IEL's (αβ-IELs) that filter luminal contents for foreign antigens or toxins and are generally responsible for signaling the presence of unwanted agents by active expression of pro-inflammatory cytokines[31,32]. The third subpopulation of surveillance lymphocytes known as γ-δ IEL's (γδ-IELs) are tasked with (1) controlling and temporizing the signaling functions of the first two subpopulations of αβ-IELs; (2) defend against microbial invasions; (3) support epithelial cells; and (4) focal elaboration and feedback secretion of transforming TGFβ[26-30]. γδ-IELs are subject to direct modulation by neighboring epithelial cells. The communications between IEL's and epithelial cells are conducted *via* cytokines[28,29,33,43-45].

Submucosal immune cells, namely mast cells, are stimulated (up-regulated) by pro-inflammatory cytokines released from upregulated IELs. Up-regulated mast cells release pro-inflammatory cytokines that in turn affect (or up-regulate) sub-mucosal neurons[35-42]. Submucosal neurons up-regulate by pro-inflammatory cytokines from IEL-stimulated mast cells then elaborate and release neuron-derived cytokines and effector substances like substance-P, vasoactive intestinal protein and neurokinins[23,31,32,34].

In turn, neuro-cytokines and effector substances released by up-regulated neurons can (1) stimulate epithelial cells to secrete fluids[23,24,46]; (2) stimulate sub-mucosal muscularis and the circular muscles of the gut to contract while simultaneously causing the longitudinal muscles to relax[23-25,47,48], (actions that result in intestinal cramping and bloating); and (3) stimulate capillary vessels to expand and increase their flow[23,24]. Additionally, stimulated sub-mucosal sensory neurons release pain substances within the sub-mucosa and into the bloodstream; they also transmit up-regulating neuronal signals outside the GI tract into dorsal root ganglia of the spine[23,24,49,50] to affect segments of the GI that are proximal and distal to the area of IEL activation.

These mucosal-mediated actions are defensive and lead to a "functional mucosal syndrome", a syndrome wherein the clinical symptoms of nausea, vomiting, pain[24,49], colic, ileus[47,48], even diarrhea[50-52] arises from mucosal mediated defensive actions provoked by antigen stimulated firing of αβ-IELs. These actions structure substantially the mucosal immuno-neuronal physiology that is indirectly affected by HPS on the instance of its contact with the mucosa.

These defensive functions of the epithelium are led by an exaggerated presence of pro-inflammatory cytokines that are secreted out of balance relative to the presence of anti-inflammatory cytokines. Activated growth factors similar to fibroblast growth factor, EGF, and TGF[53-57] are tasked with restoring cytokine balance. The consequence of disproportionate concentration of pro-inflammatory cytokines is a feedback secretion of growth factors, and more importantly, a feedback increased expression of growth factor receptor sites on nascent enteric epithelial cells[58]. Activated growth factors, once inserted into their tyrosine kinase membrane receptors, spawn the release of anti-inflammatory cytokines, with a feedback reversal of expressed pro-inflammatory cytokines[58] as well as re-epithelialization of the mucosa[55].

HPS facilitate local engagement of mucosal growth factors

HPS engagement of the mucosal surface may lead to focal movement growth factors, which facilitate conformational changes to enable their insertion into tyrosine membrane receptors[53]. In this way HPS supports the "immuno-balancing" efforts of growth factors by direct physical engagement of growth factors[53,54]. Thusly, HPS accelerates a growth factor-dependent correction of "cytokine imbalance", reversing "functional mucosal syndrome", with the reversal of nausea, vomiting, diarrhea, ileus, cramping, and bloating[47,48,50,52]. Active engagement of growth factors by HPS accelerates healing of erosions and ulcerations[53,54,56].

Potency-enhanced sucralfate or HPS appears useful for treatment and prevention of chemo-radiation induced mucositis in both the upper and lower GI tract. Given that severe cases of mucositis lead to dehydration, systemic infections and unwanted reduction or postponement of optimal cancer treatment this observation in reported here could be significant. Disciplined investigations on the use of HPS in patients with mucositis are necessary to assess reproducibility of this observation and to establish efficacy and safety. The suggested mech-

anism of action is testable by multi-array cytokine analysis of mucosal biopsies prior to, during and following treatment with HPS. Obviously, like HPS, any other non-systemic polysaccharides suitable for potency-enhancement could be investigated for efficacy in the treatment of oral and alimentary mucositis.

COMMENTS

Background

An imbalance favoring pro-inflammatory cytokines over anti-inflammatory cytokines is likely involved in the disease process of chemo-radiation induced oral and alimentary mucositis. Cancer patients suffering from mucositis have limited therapeutic support options. As a result poorly treated mucositis can lead to suboptimal cancer treatment, dehydration, costly re-hospitalizations and untimely deaths.

Research frontiers

In 2012 the Mucositis Study Group of the Multinational Association of Supportive Care in Cancer/International Society for Oral Oncology reviewed 64 clinical studies involving 11 interventions for mucositis. They found only one intervention adequate for guideline recommendation. Evidence supported limited use of recombinant human KGGF-1 (palifermin) for prevention (but not treatment) of oral mucositis if given three d prior to conditioning and three d following autologous stem cell transplantation in hematological malignancies. Inconclusive evidence prohibited guideline recommendations for any other intervention. Standard potency sucralfate was not recommended.

Innovations and breakthroughs

High potency sucralfate (HPS) is new. It is a suspension of standard potency sucralfate in a cationic solution of multi-dentate chelators. HPS hyper-concentrates sucralfate on the mucosal lining such that 3 h following its administration, the surface concentration of sucralfate remains 7-23 fold greater than otherwise expected - 7 fold greater on normal mucosal and 23 fold greater on ulcerated lining. Sucralfate of standard potency binds mucosal growth factors, yet fails to demonstrate substantial clinical effects. However the use of HPS in this patient resulted in simultaneous prevention and treatment of oral and intestinal mucositis. It is assumed therefore that there is an augmented interaction between HPS and mucosal growth factors.

Applications

The use of HPS infers that there are additional mechanisms of action for sucralfate than previously thought. These would include immuno-modulation centered on engagement and activation of mucosal growth factors as well the depolarization of ion-gated nociceptors resulting in rapid relief of mucosal pain. There may be other applications of HPS particularly in clinical scenarios dependent on epithelial healing and repair.

Peer review

This paper explores HPS may directly or indirectly facilitate an immunomodulatory mechanism involving accelerated growth factor activation, which may be a new target for therapeutic intervention in such patients. It is an interesting and very well written article.

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BRIEF ARTICLE

Examining the relationship between physical fitness and spiritual fitness in cancer patients: A pilot study

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Abstract

AIM: To examine the relationship between spiritual fitness and overall physical fitness, and their resulting impact on feelings of depression and anxiety in individuals being treated for cancer.

METHODS: Thirty patients completed the McGill Quality of Life questionnaire and the Spiritual Fitness Assessment survey, and were asked to classify themselves as "Religious" or "Non-Religious". After the questionnaires were completed, each patient underwent a comprehensive fitness assessment, which included assessments for VO_{2max}, muscular strength and endurance, flexibility, and body composition, as well as height, weight, and resting heart rate and blood pressure. The data collected were averaged and analyzed using a one-way ANOVA test at the 0.05 level of significance.

RESULTS: Of the 30 participants, 17 classified themselves as "religious" (*R*) and 13 classified themselves as "non-religious" (*NR*). The *R* group had a higher body fat percentage and a lower VO_{2max} than the *NR* group. However, these results were not significant. It was also determined that the *R* group scored themselves significantly higher than the *NR* group on the Spiritual Fitness questionnaire, but reported significantly higher levels of depression and anxiety than their non-religious counterparts.

CONCLUSION: Health beliefs did not necessarily back up health practice; specifically, those respondents who classified themselves as "religious" reported that their beliefs positively influenced their health behaviors, yet physiological and psychological data did not support this claim.

Key words: Cancer; Exercise; Spirituality; Health practice; Health beliefs; Fitness; Anxiety

Core tip: The purpose of this study was to examine the relationship between spiritual fitness and overall physical fitness, and their resulting impact on feelings of depression and anxiety in individuals being treated for cancer. Thirty participants completed a quality of life and a spiritual fitness survey, and performed a comprehensive fitness evaluation. It was determined that health beliefs did not necessarily back up health practice; specifically, those respondents who classified themselves as "religious" reported that their beliefs positively influenced their health behaviors, yet physiological and psychological data did not support this claim.

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INTRODUCTION

A cancer diagnosis can affect an individual's core as-

sumptions regarding life trajectory, beliefs about the self, control, self-worth, and the existential. Oftentimes such a diagnosis leads an individual to a process of spiritual and emotional transformation[1]. It is estimated that around 58% of cancer patients experience depression[2] and approximately 23% suffer from anxiety[3]. However, a positive correlation has been found between spirituality and emotional adjustment to cancer[4,5], indicating that spirituality plays a significant role in helping patients deal with their thoughts of mortality[4].

Similarly, research documents several positive physiological and psychological changes for cancer survivors who participate in structured exercise programs. These include improvements in VO_{2max}[6], muscular strength and endurance[7], and reduced levels of fatigue[8-12], anxiety[13,14], and depression[13-15]. As such, the American Cancer Society recommends that exercise serve as an important part of an individual's cancer care plan, asserting that exercise will improve an individual's feelings of control and hope[16]. However, published reports indicate that most (85%) of the cancer population are not currently meeting these recommendations for exercise[17].

Along with the beneficial effects of habitual physical activity, research studies have shown religious involvement to positively impact mortality risk[18], health status, mental and physical well-being[19], and a sense of self-efficacy[20]. The positive impact appears to extend to all genders, races, and socioeconomic categories[21,22]. However, recent studies show that people who would classify themselves as religious individuals are more likely to be obese than their non-religious counterparts[22,23].

Now more than ever, healthy behavior choices and emotional support are being promoted in attempt to limit cancer. Traditionally, research examining the relationship between spiritual and physical fitness has focused mostly on the healthy adult population. However, in light of the physiological stress experienced by many cancer survivors, it is critical that their psychosocial needs be addressed, as well. Therefore, the purpose of this study was to examine the relationship between spiritual fitness and overall physical fitness, and their resulting impact on feelings of depression and anxiety in individuals being treated for cancer.

MATERIALS AND METHODS

Subjects

Thirty patients were recruited for participation in this investigation. The eligibility criteria included individuals who are currently undergoing cancer treatment and are able to read and write in English. Patients were recruited from local oncology offices and hospitals. All procedures were approved by the Wright State University Institutional Review Board prior to data collection.

Data collection

The 30 patients who met the eligibility criteria and agreed to participate filled out the McGill Quality of Life questionnaire and the Spiritual Fitness Assessment survey (Fletcher, D), where they were asked to classify themselves as "Religious" or "Non-Religious". Religious was defined as "having or showing belief in and reverence for God; implies both belief and practice" (The Free Dictionary). After the questionnaires were completed, each patient underwent a comprehensive fitness assessment. The fitness evaluation included assessments for VO_{2max}, muscular strength and endurance, flexibility, and body composition, as well as height, weight, and resting heart rate and blood pressure measurements.

Table 1 Subject characteristics

Characteristics		*n*
Age (yr)	40.6 ± 2.6	
Gender	Male	11
	Females	19
Type of cancer	Prostate	5
	Colon	8
	Chemotherapy	20
	Breast	17
Current course of treatment	Radiation	7
	Surgery	3

Statistical analysis

The data collected from the psychological questionnaires and the fitness assessments were averaged and analyzed using a one-way ANOVA test. All data was analyzed at the 0.05 level of significance.

RESULTS

Subjects

A total of 30 individuals (*n*, male = 11, female = 19) participated in this investigation. Of these 30 individuals, a total of 17 classified themselves as "religious" (*R*) and 13 classified themselves as "non-religious" (*NR*). Table 1 illustrates the subject characteristics, along with type of cancer and current course of treatment.

Fitness assessment data

Figure 1 presents VO_{2max} and body composition results. Although the results show a trend where the Religious group had a higher body fat percentage (R = 35.13% ± 2.4%, NR = 32.33% ± 3.4%, Figure 1), and a lower VO_{2max} (in mL/kg per minute, R = 17.25 ± 1.7, NR = 22.24 ± 1.8, Figure 1), these results were not significant.

Physiological and spiritual questionnaires

Two questions from the Spiritual Fitness Assessment were selected from the questionnaire to compare with the biometric data. The analyzed questions from the Spiritual Fitness Assessment were: "I engage in healthy behaviors to care for my body as God's temple", which received an overall mean of 2.88 ± 0.3, on a 7-point scale, from respondents; and "I draw special strength/power from God's Spirit to make health-related behavior choices and

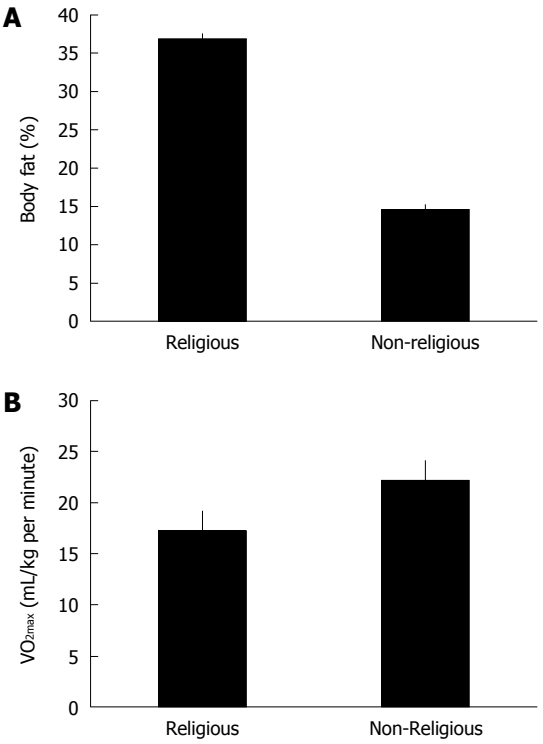

Figure 1 VO_{2max} and body composition results. A: Body fat percent; B: VO_{2max}. Values are mean ± SE.

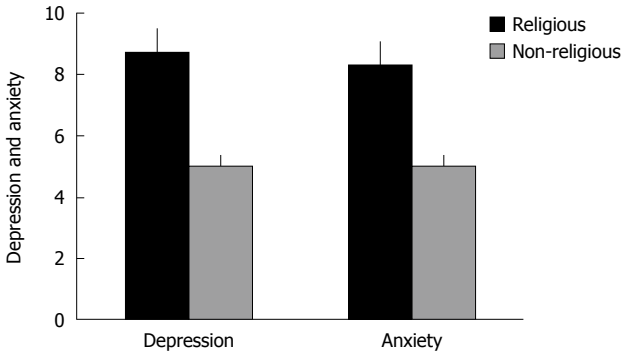

Figure 2 Depression and anxiety scores. Values are mean ± SE, on a 10-point scale.

changes in my life", which scored an overall mean of 2.78 ± 0.34 on a 7-point scale. When examined according to their respective *R*, *NR* groups, it was determined that the *R* group scored themselves significantly higher than the *NR* group on both questions (*R* = 3.77 ± 0.7, *NR* = 2 ± 0.3, *P* = 0.001; *R* = 3.44 ± 0.6, *NR* = 2 ± 0.2, *P* = 0.001, respectively).

Two questions on the McGill Quality of Life questionnaire were also analyzed. Patients were asked to indicate on a scale of 1-10 the level of depression and anxiety they have experienced over the last 2 d. Figure 2 presents the results from these surveys. It was determined that individuals in the *R* group experienced significantly higher levels of depression (*R* = 5.25 ± 0.3, *NR* = 3 ± 0.4; *P* = 0.05) and anxiety (*R* = 5 ± 0.25, *NR* = 3 ± 0.6; *P* = 0.03) than their *NR* counterparts.

DISCUSSION

The purpose of this study was to examine the impact of spiritual fitness on overall physical fitness and feelings of depression and anxiety in individuals being treated for cancer. A major finding of this investigation was that health beliefs did not necessarily back up health practice. Although those who classified themselves as "religious" reported that their beliefs positively influenced their health behaviors, physiological and psychological data did not support this claim.

Physical fitness and religion

In the present investigation, religious individuals reported that their belief in God enabled them to make health-related behavior choices and changes in their life. However, when compared to the *NR* group, they had a higher percentage of body fat and a lower VO_{2max}. This finding is consistent with previous investigations, which report an inverse relationship between religious involvement and fat intake[24] and activity levels[25]. Because of their unique role in spiritual guidance, communication, and social support, churches can play an important part in health promotion efforts. Whitt-Glover *et al*[26] found that a faith-based physical activity intervention was successful at increasing physical activity among sedentary adults. Exercise programs that incorporate faith-based practices may appeal to religious individuals (*i.e.*, modest clothing, noncompetitive atmosphere), and provide an alternative strategy for increasing physical activity[27].

Coping

The present study found that religious individuals treated for cancer had higher rates of anxiety and depression than their non-religious counterparts. This finding is not in agreement with a considerable body of literature about the role of religion and coping with morbidity and mortality. Traditionally, research has showed that religious involvement is associated with a decrease in anxiety in both healthy populations[28], and in those battling cancer[29-33].

Appropriate coping techniques are important in combatting the anxiety associated with cancer. Treatment for anxiety typically begins with giving the patient adequate information and support, then developing coping strategies that suit the needs of each patient. Research on the role of religion in helping patient cope has traditionally focused on the behavioral variables of the individual, including church affiliation and attendance[34]. However, an investigation by Bowie *et al*[35] reported that it was the combination of attending church and accepting its teachings that led to lower levels of anxiety than simply church attendance alone. In other words, patients who fully accept their churches teachings on divine healing tend to report less anxiety than those who merely attended a church. Along those lines, a recent report indicated that individuals who claim to be "spiritual" but lack an allegiance to a specific religion may actually be more likely

to experience mental health problems[36]. Thus, certain forms of religious coping affect anxiety differently in cancer patients.

Three different ways religion is involved in coping with major life stressors have been identified: (1) "self directing" coping: where it is assumed that God has provided individuals with the skills and resources to handle their problems; (2) "deferring" coping: which involves the delegation of the responsibility to God, while individuals wait passively on the outcome; and (3) "collaborative" coping: whereby God is defined as a partner who shares in the responsibility with individuals for problem solving[37]. Research indicates that respondents who indicate that they adhere to a deferral-oriented coping style tend to be less anxious than those who cope using self-directing and collaborative means[37].

Practical applications

These findings point to a wide gulf that presently exists between the ideal cancer care and that which is received by most Americans[38], and support a 2005 report from the Institute of Medicine, which highlighted a need to allocate more health care resources for these patients' unique needs[39]. It is imperative that resources be made available to address palliative care, addressing the role of lifestyle and behavior change in improving the health and function of cancer survivors[40,41]. Research suggests that physical activity, nutrition, and emotional support are associated with decreases in feelings of depression, symptoms of late effects of treatment, and cancer relapse, as well as increased remission rates[40,41]. Therefore, efforts must be made to reach out to this unique group of individuals.

COMMENTS

Background

A cancer diagnosis can affect an individual's core assumptions regarding life trajectory, beliefs about the self, control, self-worth, and the existential. Oftentimes such a diagnosis leads an individual to a process of spiritual and emotional transformation. It is estimated that around 58% of cancer patients experience depression and approximately 23% suffer from anxiety. However, a positive correlation has been found between spirituality and emotional adjustment to cancer, indicating that spirituality plays a significant role in helping patients deal with their thoughts of mortality. Thus, the purpose of this study was to examine the relationship between spiritual fitness and overall physical fitness, and their resulting impact on feelings of depression and anxiety in individuals being treated for cancer.

Research frontiers

The important areas in the research field related to this article are any areas that would analyze the quality of life of an individual undergoing cancer treatment. A host of disciplines would be interested to read about how exercise and spirituality can impact anxiety and depression.

Innovations and breakthroughs

A major finding of this investigation was that health beliefs did not necessarily back up health practice. Although those who classified themselves as "religious" reported that their beliefs positively influenced their health behaviors, physiological and psychological data did not support this claim.

Applications

These findings point to a wide gulf that presently exists between the ideal cancer care and that which is received by most Americans, and support a 2005 report from the Institute of Medicine, which highlighted a need to allocate more health care resources for these patients' unique needs. It is imperative that resources be made available to address palliative care, addressing the role of lifestyle and behavior change in improving the health and function of cancer survivors. Research suggests that physical activity, nutrition, and emotional support are associated with decreases in feelings of depression, symptoms of late effects of treatment, and cancer relapse, as well as increased remission rates. Therefore, efforts must be made to reach out to this unique group of individuals.

Terminology

Religious: having or showing belief in and reverence for God; implies both belief and practice. Physical Fitness: good physical condition; being in shape or in condition; the state of good health. Anxiety: a feeling of worry, nervousness, or unease, typically about an imminent event or something with an uncertain outcome. Depression: severe despondency and dejection, accompanied by feelings of hopelessness and inadequacy.

Peer review

The peer reviewer read with great interest the manuscript, this manuscript is interesting and adds valuable information to this field.

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BRIEF ARTICLE

Evaluation of three methods for detection of methicillin-resistant *Staphylococcus aureus*

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Abstract

AIM: To evaluate GenoType methicillin-resistant *Staphylococcus aureus* (MRSA) Direct assay and cultivation for the identification of MRSA by using *mecA* polymerase chain reaction (PCR) as the "gold standard" assay.

METHODS: In total of 61 nasal specimens from patients at the intensive care unit were studied by GenoType MRSA Direct test, conventional culture method and automated bacterial identification system. The results of GenoType MRSA Direct assay were compared to conventional culture method the identification of MRSA and *mecA* gene PCR as the "gold standard" method. The sensitivity, specificity, positive predictive value and negative predictive value were calculated.

RESULTS: In total, 61 specimens were studied. Fifty-four specimens (88.5%) were negative by all three methods. Six swabs (9.8%) were found positive by GenoType MRSA Direct test, conventional culture method and automated bacterial identification system. The presence of *mecA* in these strains was confirmed by PCR. One swab sample was negative for culture methods but MRSA and *mecA* gene were detected by GenoType MRSA Direct test and *mecA* PCR respectively. GenoType MRSA Direct test had a sensitivity of 100% (6/6) and a specificity of 100% (55/55), with a positive predictive value of 100% and a negative predictive value of 98%. Culture method of MRSA had a sensitivity of 83.3% (5/6) and a specificity of 98.2% (55/56).

CONCLUSION: It was found that the GenoType MRSA Direct assay, which is a rapid and accurate test, is of the same sensitivity and specificity with mecA PCR. The GenoType MRSA Direct assay can be a better tool for rapid and accurate detection of MRSA in diagnostic laboratories.

Key words: Culture; Methicillin-resistant *Staphylococcus aureus*; Molecular assays

Core tip: For the identification of methicillin-resistant *Staphylococcus aureus* (MRSA), GenoType MRSA Direct assay and cultivation were evaluated by using mecA polymerase chain reaction (PCR) as the "gold standard" assay. Fifty-four specimens (88.5%) were negative by all three methods. Six swabs (9.8%) were found positive by GenoType MRSA Direct test, conventional culture method and automated bacterial identification system. The presence of *mecA* in these strains was confirmed by PCR. One swab sample was negative for culture methods but MRSA and *mecA* gene were detected by GenoType MRSA Direct test and *mecA* PCR respectively. It was found that the GenoType MRSA Direct assay, which is a rapid and accurate test, is of the same sensitivity and specificity with mecA PCR.

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INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become increasingly prevalent worldwide. Since its discovery during the 1960s, MRSA has emerged as a common cause of nosocomial infection. Infections caused by MRSA is one of the major sources of morbidity and mortality nosocomial infections especially in the intensive care units (ICU). Prevention of nosocomial infections caused by MRSA in ICU has been recommended for several years[1,2]. The spread of MRSA can be controlled by effective preventive measures and to limit this spread, a rapid and sensitive test for detection of MRSA colonization is required. However, conventional tests for the identification of MRSA require at least 48 h to be completed[3]. In recent years, there has been a growing emphasis on the use of molecular methods to detect not only just infectious agents but also antimicrobial-resistance genes carried by microorganisms[4]. Several DNA based tests have been developed for the rapid detection of MRSA[3].

Methicillin resistance in *Staphylococcus aureus* (*S. aureus*) is mediated by the production of an altered penicillin-binding protein, PBP 2a[5]. The mec gene complex regulates the production of PBP 2a. The detection of the *mecA* gene or of PBP 2a provides much more accurate detection of methicillin resistance in *S. aureus*[5,6]. *MecA* gene detection tests based on polymerase chain reaction (PCR) are considered as the gold standard for methicillin resistance[5].

Screening of the patients with risk factors for MRSA carriage is important for a successful MRSA control policy. At our hospital, infection control precautions are taken immediately after a positive MRSA result becomes available from diagnostic and surveillance specimens. Since molecular methods are rapid, with turnaround times of 2 to 4 h, these tests are able to improve the utilization of infection control resources. We compared GenoType MRSA Direct assay and culture for identification of MRSA using PCR for mecA as the "gold standard" assay.

MATERIALS AND METHODS

A total of 61 consecutive patients were screened for MRSA. Of the patients, 32 (52.4%) were female and 29 (47.5%) were male. Ethical approval was received from Ataturk Training and Research Hospital Ethics Committee.

Specimen collection and processing

Nasal specimens were obtained from patients at ICU. Two concurrent specimens were obtained from each site swabbed. First swab was used for culture and second swab was used for molecular assays. Swabs were transported at room temperature and processed within 1 to 3 h of collection.

Bacteria isolation

The swabs were inoculated on blood agar plates directly on the day of receipt of the swab, incubated at 35 ℃ in O_2, and read after 24 and 48 h. A colony suggestive of Staphylococcus was confirmed as *S. aureus* by using a tube coagulase and DNase test, while methicillin resistance was confirmed with cefoxitin susceptibility testing according to the Clinical and Laboratory Standards Institute method[7]. The Phoenix (Becton Dickinson, Sparks, MD, United States) was used for confirmation of strains. Control strains, MRSA strain (ATCC 43300), and methicillin-susceptible *S. aureus* (ATCC 25923) were used in all tests.

GenoType MRSA direct assay

DNA extraction and amplification: The swabs were processed using the GenoType MRSA Direct (Hain Lifescience, Nehren, Germany) method. According to the manufacturers' recommendations, the swabs were washed in 300 μL of lysis buffer before DNA extraction. Bacterial DNA was released by incubation of the lysis buffer for 10 min at 95 ℃, followed by centrifugation for 5 min at 6000 *g*. Portions (5 μL) of the supernatant were used for amplification. In brief, 45 μL of primer nucleotide mix (provided with the kit), $MgCl_2$ to a final concentration of 2.5 mmol/L and 1 U of HotStart Taq polymerase (Qiagen, Hilden, Germany) were added, followed by amplification on a PE 9700 thermocycler (Applied Biosystems, Weiterstadt, Germany) for 15 min at 95 ℃, 35 cycles of 95 ℃ for 30 s, 55 ℃ for 40 s and 72 ℃ for 40 s, and a final extension at 70 ℃ for 8 min. Each run included a negative control sample to demonstrate the absence of contaminating DNA. The sensitivity of amplification and hybridisation was monitored using an internal control.

Hybridization protocol: Briefly, the assay uses a specific oligonucleotide probe, targeting the SCCmec chromosomal cassette of MRSA immobilized on membrane strips. During the detection process PCR amplicons hybridise with this probe. Hybridization and detection were performed in an automated washing and shaking device (Profiblot; Tecan, Maennedorf, Switzerland). PCR products (20 μL) were mixed for 5 min with 20 μL of denaturing reagent (provided with the kit) at room temperature in separate troughs of a plastic tray. After addition of 1 mL of pre-warmed hybridization buffer, the membrane strips in the kit were added to every trough. Hybridization was at 45 ℃ for 30 min, followed by two washing steps at 45 ℃ for 30 min with 1 mL of pre-warmed stringent wash solution. Streptavidin-conjugated alkaline phosphatase and the appropriate substrate were added for colourimetric detection of hybridised amplicons. After final washing, the strips were air-dried and fixed on a data sheet. DNA isolation, amplification and hybridisation, were monitored using an internal control to improve the reliability of the test.

MecA gene PCR

The following primers were used: M1 (TGGCTATCGTGTCACAATCG) and M2 (CTGGAACTTGTTGAGCAGAG) that amplify a 310-bp fragment of the *mecA*

gene, which codes for the PBP 2a protein. The mixture for PCR consisted of 5 μL of PCR buffer 10 (final concentration: 50 mmol/L KCl, 0.01% gelatin, 10 mmol/L Tris-HCl; pH 8.3), 1.5 mmol/L $MgCl_2$, 0.1 mmol/L dNTP (dATP, dCTP, dGTP and dTTP), 0.4 pmol/mL of each of the specific primers, 2 U of tag polymerase and 5 μL of the template to get a final reaction volume of 50 μL. All components were mixed in polystyrene tubes and subjected to amplification temperatures of the nucleic acid in a thermocycler. DNA from the methicillin resistant *S. aureus* reference strain (ATCC 43300) was used as positive control of the reaction and sterile bi-distilled water as negative control.

The mixture was initially denatured at 94 ℃ for 5 min and then underwent 35 cycles of: 30 s at 94 ℃, 30 s at 52 ℃ and 30 s at 72 ℃. The reaction was finished with 5 min at 72 ℃. The amplification products were detected with agarose gel electrophoresis (2% in TBE 0.5X buffer) at 100 V for 35 min.

Statistical analysis

The results of GenoType MRSA Direct assay were compared to conventional culture method the identification of MRSA and *mecA* gene PCR as the "gold standard" method. The sensitivity, specificity, positive predictive value and negative predictive value were calculated.

RESULTS

In total, 61 specimens were studied. Fifty-four specimens (88.5%) were negative by all three methods. Six swab samples (9.8%) were positive by conventional culture method and automated bacterial identification system (Table 1). The presence of *mecA* in these strains was confirmed by PCR and GenoType MRSA Direct test. Although one swab was negative by conventional culture method and automated bacterial identification system, it was positive for m*ecA* gene detected by GenoType MRSA Direct test and *mecA* PCR respectively. The results of 61 specimens studied were shown in Table 1. GenoType MRSA Direct test had a sensitivity of 100% (6/6) and a specificity of 100% (55/55), with a positive predictive value of 100% and a negative predictive value of 98%. Culture method of MRSA had a sensitivity of 83.3% (5/6) and a specificity of 98.2% (55/56). Images of *mecA* PCR and GenoType MRSA Direct test are shown in Figures 1 and 2.

DISCUSSION

The prevalence of MRSA carriage on hospital admission is important in determining the effect of implementing any screening policy. Standard culture methods require at least 24 to 48 h for the recovery and identification of *S. aureus* and additional confirmatory tests[8] or susceptibility testing methods to determine methicillin resistance. Genotype MRSA direct test has been completed in approximately 4 h for detecting MRSA in the present study. Early and specific diagnosis of MRSA infections is significant in preventing their spread. Delays in detection of MRSA lead to the increased transmission of MRSA among patients, higher numbers of MRSA infections, and increased hospital costs[3]. A rapid and reliable test for the identification of MRSA would be desirable so that effective therapy could be initiated immediately. In recent years, there has been a growing emphasis on the use of molecular methods to detect not just infectious agents but also antimicrobial-resistance genes carried by microorganisms[4]. A study investigating the value of rapid diagnostic tests for MRSA when used for admission screening to a critical care area reported a reduction in the incidence of transmission of MRSA from 13.89/1000 patient days to 4/1000 patient days[9].

PCR tests are valuable for the rapid detection of MRSA carriers[10]. Conventional culture based detection methods for MRSA are time-consuming which leads to delayed isolation. Rapid molecular detection assays such as conventional PCR, real-time PCR and gene probe hybridization assays. Real-time PCR, has improved the sensitivity, and specificity, enables detection of resistance in

Table 1 Results for the detection of methicillin-resistant *Staphylococcus aureus*

n	Genotype MRSA Direct test	Culture	MecA PCR
54	-	-	-
6	+	+	+
1	+	-	+
Total = 61			

MRSA: Methicillin-resistant *Staphylococcus aureus;* PCR: Polymerase chain reaction.

Figure 1 Agarose gel electrophoresis for mecA (310 bp) gene. Lanes 1 and 38: Molecular weight ladder; Lane 2: Negative control; Lanes 4, 10, 14, and 20: Positive clinical isolates; Lane 37: Positive control; Other lanes: Negative clinical isolates.

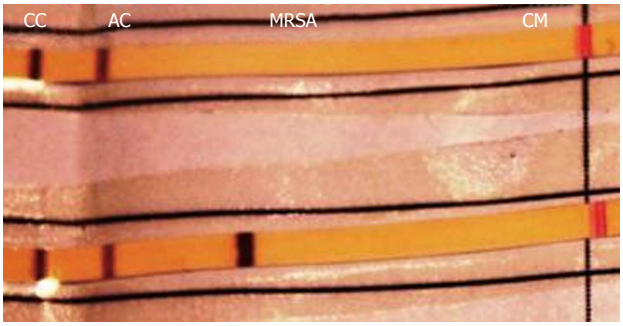

Figure 2 Image of positive and negative sample in GenoType methicillin-resistant *Staphylococcus aureus* Direct test. CC: Conjugate control; AC: Amplification control; MRSA: Methicillin-resistant *Staphylococcus aureus*; CM: Colored marker.

a shorter time and lower risk of contamination than conventional PCR[11,12]. IDI-MRSA and the genotype MRSA tests can detect MRSA within a few hours directly from screening swabs with good sensitivity and specificity of 81% to 92% and 93% to 98%, respectively[10]. GenoType MRSA Direct test was evaluated to detect for the rapid detection of MRSA from nasal specimens in the present study. We evaluated the results of GenoType MRSA Direct test with the results obtained from conventional culture assay and PCR as the gold standard. We found a sensitivity of 100% and a specificity of 100%, with a positive predictive value of 100% and a negative predictive value of 98% with three tests. Our results were similar previous studies[13-15]. Warren *et al*[13] used a commercially available real-time PCR kit to detect MRSA directly from nasal swabs of 288 patients. They reported a sensitivity of 91.7%, and a specificity of 93.5%, with a positive predictive value of 82.5% and a negative predictive value of 97.1%. Similar results were reported by Huletsky *et al*[14,15] with the same system from 331 nasal swab specimen, with a sensitivity of 100%, specificity of 96.5%, with a positive predictive value of 89.4% and a negative predictive value of 100%.

Rising colonization rates lead to increased infection rates in the community and in hospitals. It has also been reported that rapid detection of carriage has an important role to play in such a "search-and-destroy" strategy[16,17]. van Hal *et al*[18] compared the relative sensitivities and specificities of the IDI-MRSA and GenoType MRSA Direct assays and three selective MRSA agars, MRSA ID, MRSA*Select*, and CHROMagar MRSA, with swabs from the three most commonly screened sites, *i.e.*, the nose, groin and axilla. They informed that IDI-MRSA was the most sensitive method for the detection of MRSA with nasal swabs, with 90% sensitivity. GenoType MRSA Direct test had a sensitivity of 69%. However, Holfelder *et al*[3] found the sensitivity 94.5% of GenoType MRSA Direct test in their study. Swabs from 242 patients at risk for MRSA carriage were analysed by standard culture method and the PCR assay. They reported that the GenoType MRSA Direct assay provides a rapid, sensitive and specific method, in comparison with selective culture, for direct detection of MRSA in clinical swab specimens.

Tokue *et al*[6] tested *mecA* gene in 58 clinical isolates by the PCR and Southern blot analyses. Six PCR-positive strains were classified as methicillin susceptible by the conventional susceptibility test. They reported that the PCR assay appears to be more reliable than routine susceptibility testing. In the present study, one swab was negative for culture method but MRSA and *mecA* gene were detected by GenoType MRSA Direct assay and *mecA* PCR respectively. However, the broad use of MRSA PCR assays is hampered by high costs for PCR[19]. PCR tests are valuable for the rapid detection of MRSA, but high costs require the careful evaluation of their use. In patient populations with low MRSA endemicity, the broad use of PCR may not be cost-effective. But the rapid detection of MRSA carriers is important with low MRSA prevalence, since MRSA control is easiest when rates are still low, and maximal efforts should be made to maintain such epidemiology[10]. Metan *et al*[12] reported that the molecular assays would be appropriate for tertiary hospitals considering the upfront costs and requirement of expert laboratory staff.

Although conventional tests for identification of MRSA require at least 48 h, the GenoType MRSA Direct assay has rapid turnaround time of 4 h. This assay provides same day results and reduces the isolation time required for patients at risk of MRSA carriage. te Witt *et al*[20] emphasized that, nucleic acid-amplification techniques offer clear benefits over traditional culture-based assays, in particular, a reduced time to identification and an improved specificity and sensitivity. Luteijn *et al*[21] reported that it was a significantly higher sensitivity was found for the PCR in the recent article. Continual monitoring of clinical isolates is necessary to develop and maintain an effective strategy against *S. aureus* infection in the hospital setting[22].

As a conclusion, for the screening of MRSA in clinical swab specimens, it was found that the GenoType MRSA Direct assay, which is rapid and accurate test, is of the same sensitivity and specificity with mecA PCR in the present study. The GenoType MRSA Direct assay can be a better tool for the rapid and accurate detection of MRSA in diagnostic laboratories.

ACKNOWLEDGMENTS

Thanks to Hain Lifescience-Turkey for providing GenoType MRSA Direct test.

COMMENTS

Background

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become increasingly prevalent worldwide. The spread of MRSA can be controlled by effective preventive measures and to limit this spread, a rapid and sensitive test for detection of MRSA colonization is required. Screening of the patients with risk factors for MRSA carriage is important for a successful MRSA control policy. Since molecular methods are rapid, with turnaround times of 2 to 4 h, these tests are able to improve the utilization of infection control resources. Authors compared GenoType MRSA Direct assay and culture for identification MRSA using polymerase chain reaction (PCR) for mecA as the "gold standard" assay.

Research frontiers

Infections caused by MRSA is one of the major sources of morbidity and mortality nosocomial infections especially in the intensive care units (ICU). Infection control precautions should taken immediately after a positive MRSA result becomes available from diagnostic and surveillance specimens. The article's significance originates from its emphasis on the area of hospital infections.

Innovations and breakthroughs

In recent years, there has been a growing emphasis on the use of molecular methods to detect not just infectious agents but also antimicrobial-resistance genes carried by microorganisms. A study investigating the value of rapid diagnostic tests for MRSA when used for admission screening to a critical care area reported a reduction in the incidence of transmission of MRSA from 13.89/1000 patient days to 4/1000 patient days. Warren *et al* used a commercially available real-time PCR kit to detect MRSA directly from nasal swabs of 288 patients. They reported a sensitivity of 91.7%, and a specificity of 93.5%, with a positive predictive value of 82.5% and a negative predictive value of 97.1%. Similar results were reported by Huletsky *et al*. The present study was performed in a tertiary hospital. Metan *et al* reported that the molecular assays would be appropriate for tertiary hospitals considering the upfront costs and requirement of expert laboratory staff.

Applications

Besides their advantages like high sensitivity and specificity, molecular methods require experienced staff and laboured work. Yet, automatized molecular methods, being effective after in-house PCR, provides standardization along with an ease of use. As more advanced molecular methods are introduced, these methods will be preferred routinely.

Peer review

This article is considered to be helpful for the clinicians about predicting MRSA infections among ICU patients. This consideration appears to be approved explicitly by the clinicians.

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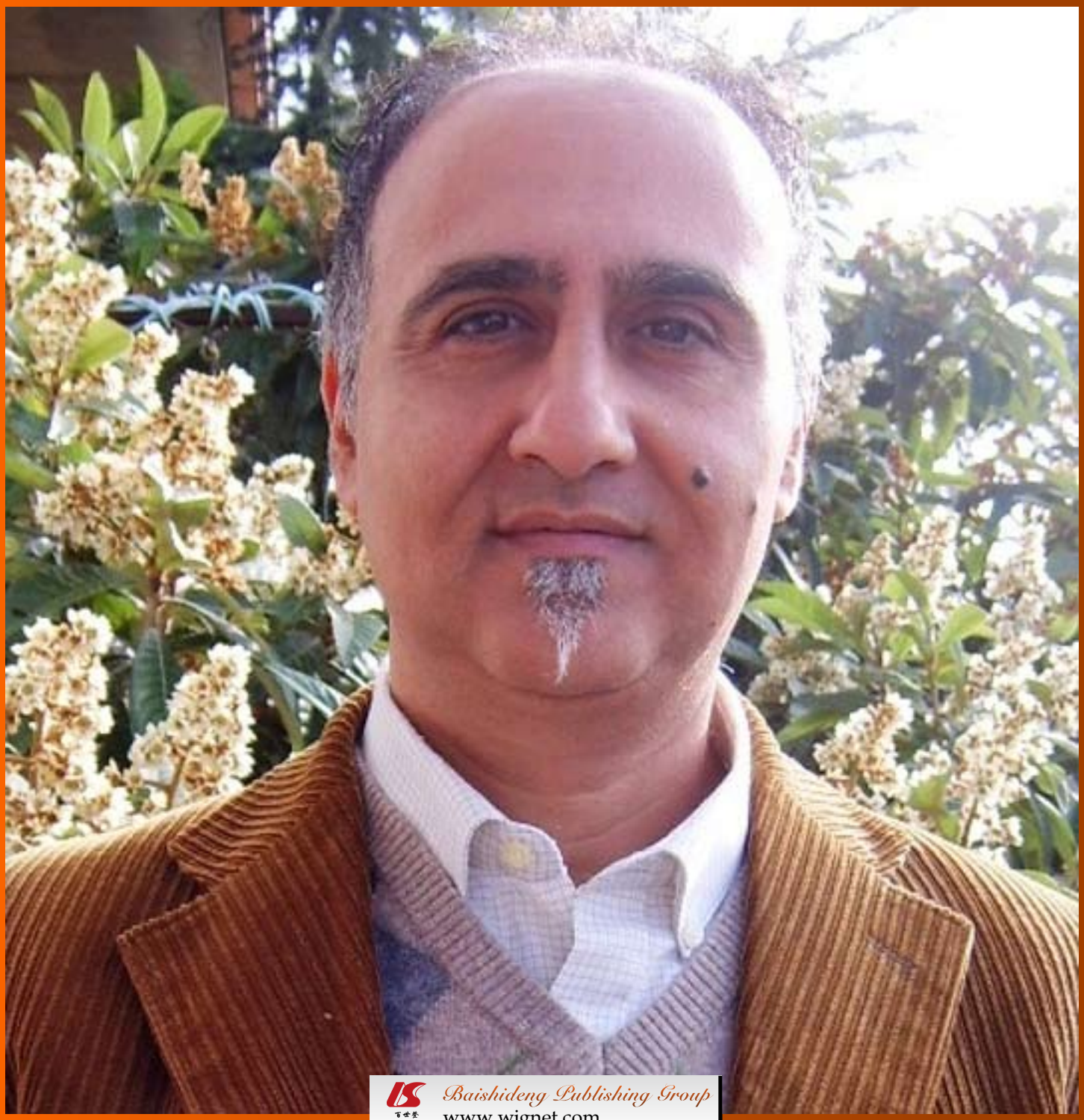

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FIELD OF VISION

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

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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.

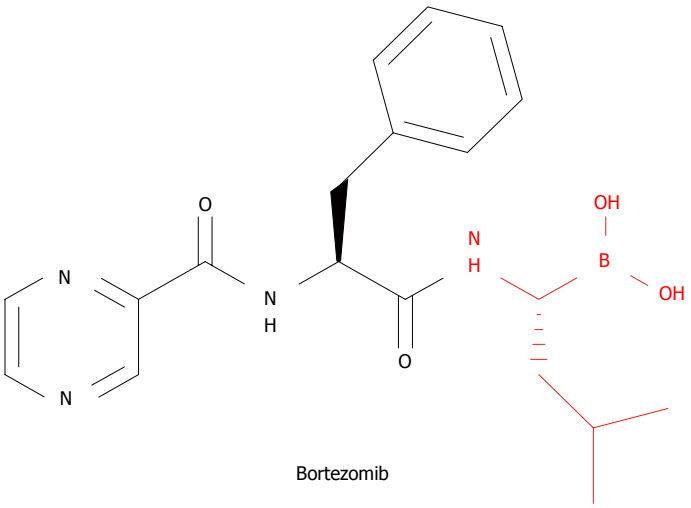
Key words: Peptide boronates; Proteasome inhibitors; Anticancer therapy; Pro-soft drug; Solid tumors

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.

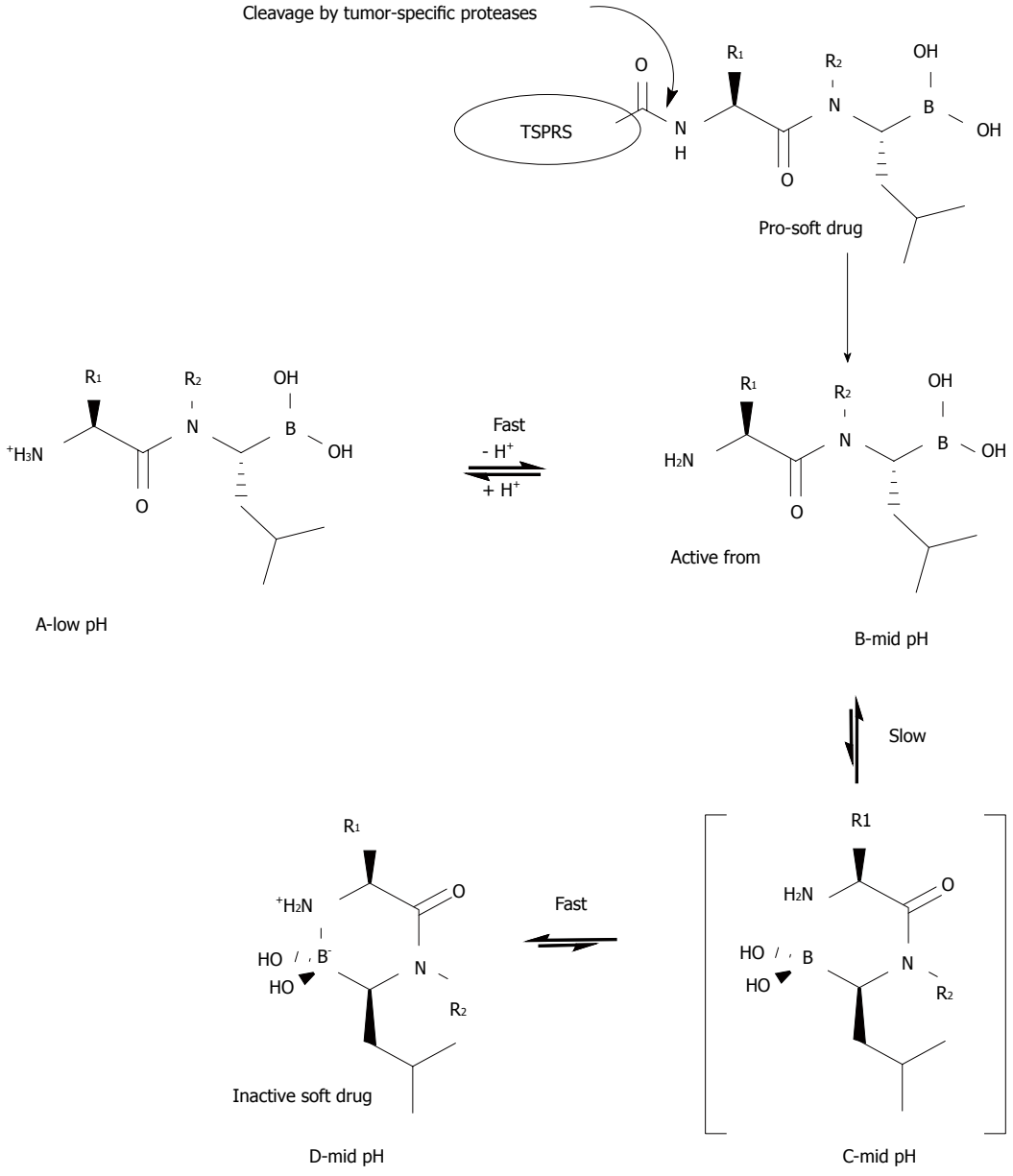


Figure 2 Activation and pH-dependent cyclization of dipeptidyl boroLeu. A tumor-specific protease recognition sequence (TSPRS) is attached to the *N*-terminus of the dipeptidyl boroLeu. The dipeptidyl boroLeu is released in its active form by a tumor-specific protease and undergoes a pH-dependent cyclization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

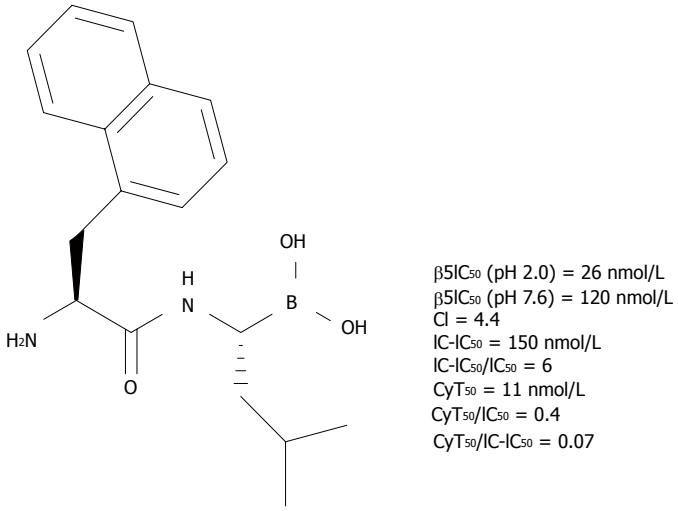


Figure 3 Structure and activity of the most interesting dipeptidyl boroLeu developed by Milo *et al*[14]. IC-IC_{50}/IC_{50} ratio → a measure of cell permeability, for which a value of 1.0 corresponds to 100%; CyT_{50}/IC_{50} ratio → the relationship between cytotoxicity and inhibition, for which a 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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REVIEW

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

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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.

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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.

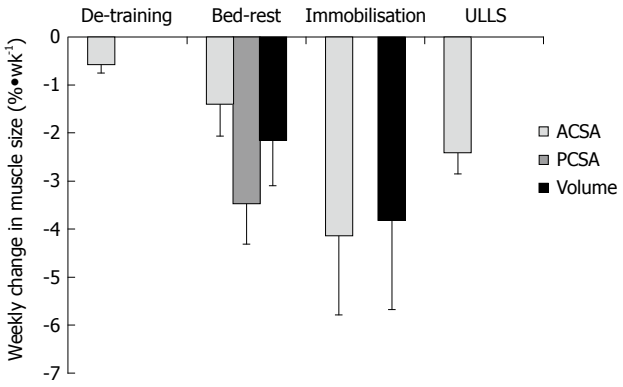

Figure 1 Relative change in muscle anatomical cross sectional area, physiological cross sectional area and volume. ULLS: Unilateral lower limb suspension; ACSA: Anatomical cross sectional area; PCSA: Physiological cross sectional area.

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

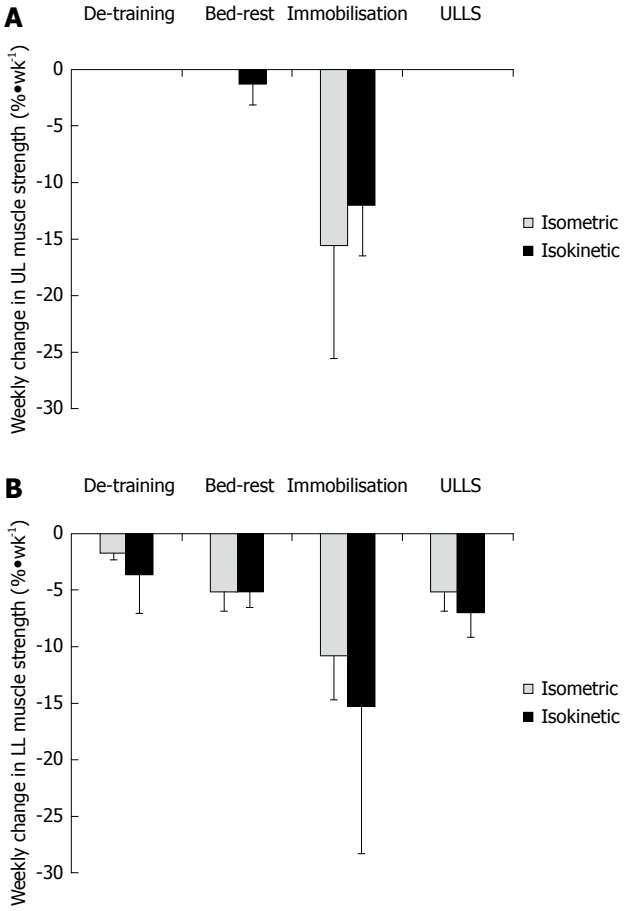

Figure 2 Relative change in isometric and isokinetic strength. A: Upper limb; B: Lower limb. ULLS: Unilateral lower limb suspension; UL: Upper limb; LL: Lower limb.

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.

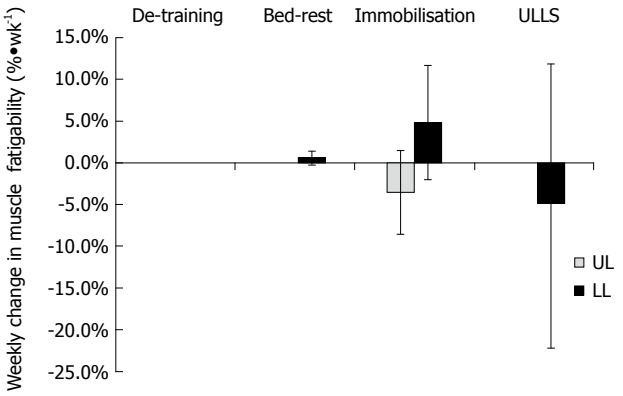

Figure 3 Relative change in muscle fatigability. ULLS: Unilateral lower limb suspension; UL: Upper limb; LL: Lower limb.

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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REVIEW

Impact of viral and bacterial infections in coronary artery disease patients

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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.

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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-

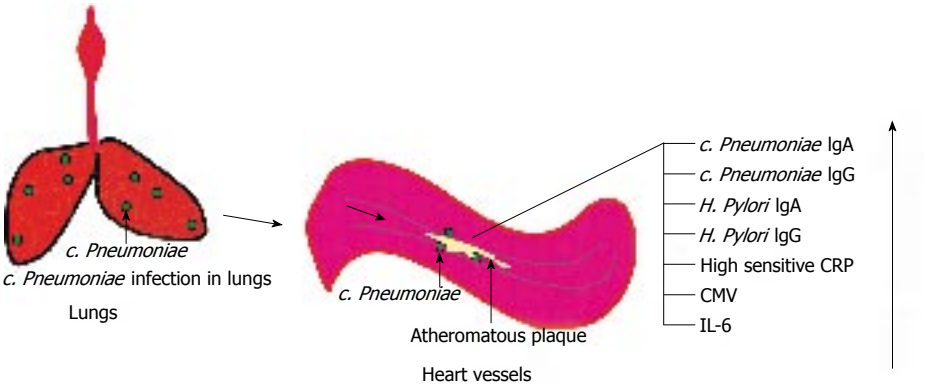

Figure 1 A schematic representation of *Chlamydia pneumoniae* infection from lungs to heart. IL: Interleukin-6; CMV: *Cytomegalovirus*; CRP: C-reactive protein; *c. Pneumoniae*: *Chlamydia pneumoniae*; *H. Pylori*: *Helicobacter pylori*.

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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REVIEW

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

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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.

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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-

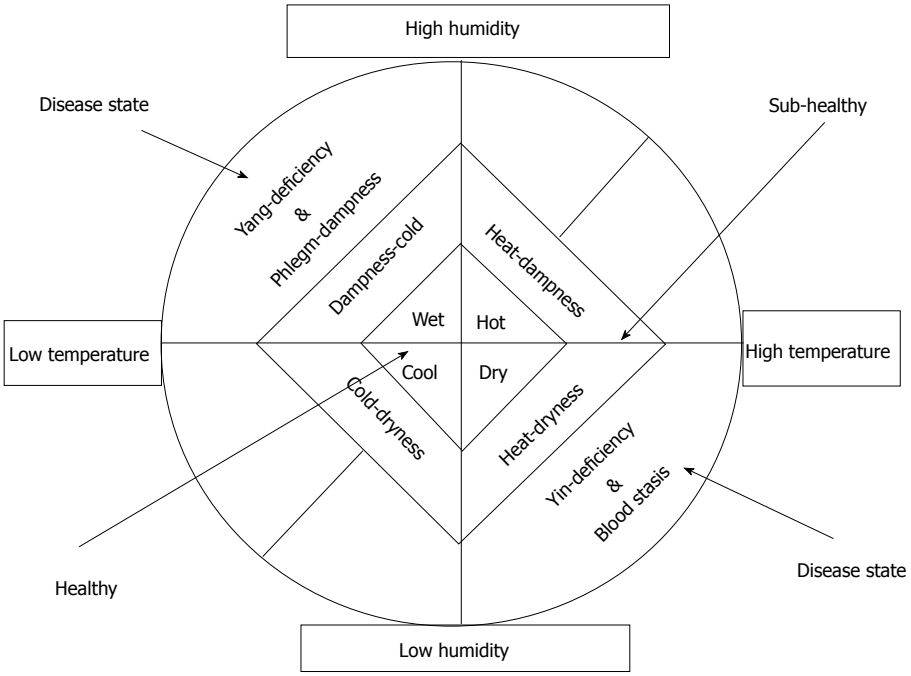

Figure 1 Diagram showing the types of body constitution in relation to health, sub-health and disease status.

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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ORIGINAL ARTICLE

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

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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.

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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

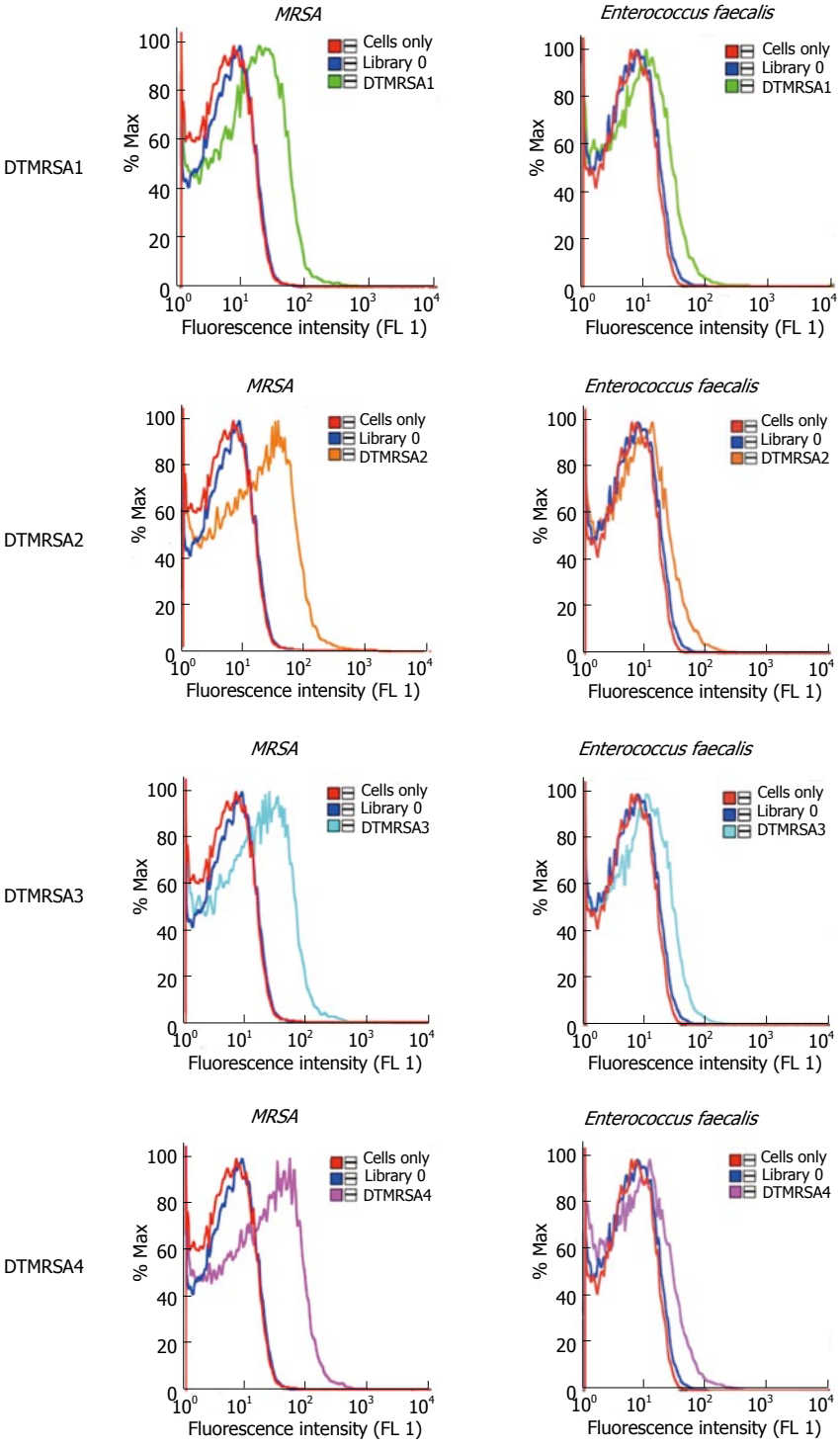

Figure 1 Flow cytometry histograms of the relevant aptamer candidates. Four aptamers show significant binding to *methicillin-resistant Staphylococcus aureus* (*MRSA*) 43300 bacteria, but not to the *Enterococcus faecalis* cell line.

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-

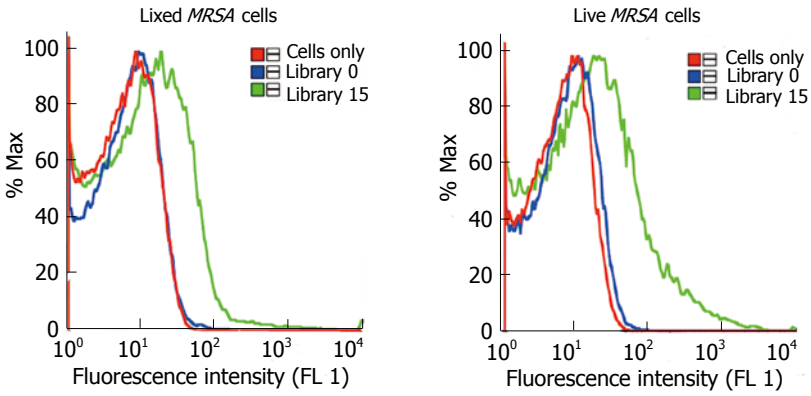

Figure 2 Comparison of the binding results between fixed and live *methicillin-resistant Staphylococcus aureus* cells. Binding assays show that results remain the same, irrespective of whether experiments are run with fixed or live cells. MRSA:*Methicillin-resistant Staphylococcus aureus*.

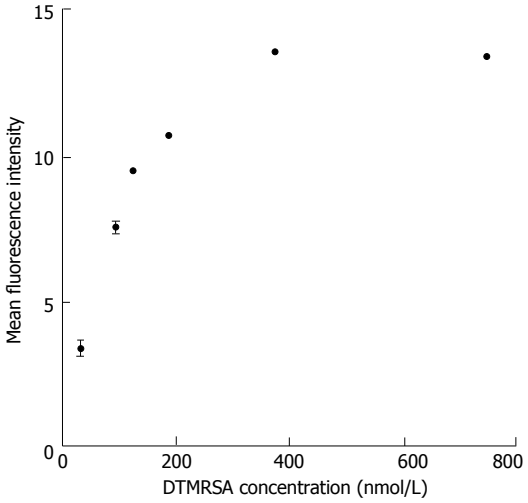

Figure 3 SigmaPlot binding curve of the aptamer DTMRSA4 used for the *Kd* value determination. *Methicillin-resistant Staphylococcus aureus* cells were incubated with various concentrations of fluorescein isothiocyanate-labeled DTMRSA4.

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.

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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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BRIEF ARTICLE

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

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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.

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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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