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UNIVERSIDADE ESTADUAL DE CAMPINAS

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"CORTISOL SANGUÍNEO E SALIVAR COMO INDICADORES DE ESTRESSE"

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Marcia Carvalho Garcia e aprovada pela Comissão Julgadora.

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Funcional e Molecular, na área de Fisiologia.

Orientadora: Profa. Dra Regina Célia Spadari

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Resumo

Hans Selye definiu *stress* como a ″resposta não específica do organismo frente a agentes ameaçadores de sua integridade″. O avanço de técnicas e métodos tanto de coleta de material biológico, quanto de análises destes tornaram esta definição polêmica. Apresentamos neste trabalho a evolução do conceito de estresse e os termos a ele associados, levando em conta que a resposta de estresse tem caráter adaptativo e visa proteger o organismo e garantir a sua sobrevivência, quando se refere a experiências de tempo limitado que o indivíduo pode superar. Por outro lado, o estresse torna-se perigoso para a saúde quando o senso de controle e o domínio são perdidos. O sistema de resposta de estresse envolve regiões cerebrais que se conectam entre si e desencadeiam estímulos por meio de dois eixos: um neural e outro hormonal aos sistemas periféricos. Os glicocorticóides participam em todas as etapas da resposta de estresse, e são, por isso, considerados como marcadores biológicos desta resposta. A determinação da concentração salivar de cortisol é, atualmente, a técnica de eleição para este fim. O objetivo deste trabalho foi avaliar se a determinação da concentração salivar de cortisol pode ser utilizada como indicador biológico de estresse relacionado a diversas atividades humanas. Determinamos a concentração sérica de cortisol em jogadores de futebol durante duas temporadas esportivas, e a concentração salivar de cortisol em atletas de basquetebol também durante duas temporadas esportivas, em estudantes durante o ano de preparo para o vestibular, além de utilizar esta técnica para investigar o índice de estresse associado ao baixo status socioeconômico. Nossos resultados mostraram que a concentração salivar de cortisol apresenta vantagens sobre a determinação da concentração sanguínea deste hormônio por ser não invasiva, ser indolor e de fácil manejo. Além disso, os dados permitiram avaliar o índice de estresse associado à prática esportiva, em duas modalidades diferentes, a uma atividade intelectual, representada pelo esforço realizado para entrar na universidade, e à condição socioeconômica adversa. Por outro lado, verificamos que cuidados devem ser tomados para garantir a adesão dos voluntários aos protocolos de coleta, uma vez que esta tende a ser baixa neste tipo de abordagem. Além disso, esta técnica é adequada para avaliar a concentração de cortisol como indicadora do índice de estresse relacionado à prática esportiva, à atividade intelectual, e o estresse associado ao

baixo status socioeconômico. Nós também concluímos que no início da temporada esportiva os atletas ficam expostos a concentrações mais altas de cortisol, embora o ritmo circadiano do hormônio seja mantido. Nos jogos matinais, mas não nos vespertinos ou noturnos foi possível estabelecer correlação positiva entre concentração de cortisol e vitória. Concluímos que, estudantes brasileiros candidatos a ingressar na Universidade apresentaram stress, depressão e ansiedade. A concentração de cortisol salivar oscilou durante o ano, com altas concentrações nos meses que os estudantes tiveram que decidir sobre a carreira a seguir e a universidade. No mês novembro, concentrações moderadas de cortisol salivar foram significativamente correlacionadas com sucesso no exame. Em pessoas de baixo status socioeconômico verificamos que os altos índices de estresse percebido e da concentração salivar de cortisol impactam negativamente em sua saúde e se correlacionam com o estresse experimentado diariamente. Considerando que esta corresponde a 30% da população brasileira, isto pode representar um grande impacto nas políticas públicas de saúde.

Abstract

Hans Selye defined *stress* as ″the organism non specific response to any threat to its integrity″. During most of the past century, Selye`s theory has been challenged by a number of scientists who discussed many of its aspects based on new data. In this work we present the evolution of stress theory and associated terms, considering that the stress response is adaptive and that it has been conserved to protect and to guarantee survival. This happens when the stressor is present for a short period, and when the subject can overcomes it. On the other hand, the stress response turns into harmful when the subject looses control over the situation. The stress system includes cerebral regions that are connected and that trigger stimuli through a neural and a hormonal axis towards the periphery, preparing the organism to the fight-or flight reaction or adaptation. Because glucocorticoids play a role in every phase of the stress response these hormones have been recognized as biological markers of the stress reaction. The aim of this work was to evaluate the salivary cortisol levels (SCL) related to several human activities and conditions. We determined the seric cortisol levels in soccer players and the salivary cortisol levels in basketball athletes during two competitive seasons, in Brazilian students during the year they prepare to enter the University, and in a group of people with low socioeconomic status. Results have shown that using salivary cortisol is better than blood levels because it is easier, painless and can be done by the subject anywhere. Moreover, this technique was suitable to evaluate the cortisol level as an indicator of the stress index related to sports practice, to intellectual activity, represented by the students fight to conquer a vacancy in the public university, and associated to low socioeconomic status (LSES). On the other hand, we also concluded that the volunteers' adhesion to the protocol of sample collection is poor and may represent a challenge to the researcher. By using this technique we concluded that in the beginning of the competitive season athletes are exposed to higher levels of cortisol. However, the cortisol circadian rhythm has been preserved during all the season. In the morning games the increase of cortisol levels are related to the game result, with higher increases correlated with victory. This does not happen for the afternoon and evening games. The Brazilian students, preparing for university entrance exams, were pronounced stressed, and have shown high levels of depression and anxiety. The salivary cortisol levels oscillate during

the year, with higher values in the months when the students are choosing their courses and university. Although the levels of salivary cortisol diminished in November, SCL were not correlated with success in the exam. We have concluded that the LSES is associated with high index stress perceived (PSI) and salivary cortisol levels which could impact negatively in health and it is related to the daily life stress experienced by LSES group. Because the LSES corresponds to about 30% of the total Brazilian population, it might have a great impact on public health policies and costs.

SUMÁRIO

INTRODUÇÃO

Na década de 1930 chega a Montreal, no Canadá, um jovem húngaro recém-formado médico na Universidade de Praga, então Thechoslováquia, para continuar seus estudos na área de endocrinologia. Torna-se polêmico ao sugerir o emprego do termo *stress* na literatura médica e científica, definido por ele, Hans Selye, como a ″resposta não específica do organismo frente a agentes ameaçadores de sua integridade″. Selye vê o conceito de *stress* sendo difundido pelo mundo e é, ao mesmo tempo, questionado quanto ao termo utilizado. Defende-se ao explicar que, no início dos seus estudos não dominava o inglês, e não distinguiu *stress* (pressão) de *strain* (esforço). ″O melhor seria que o fenômeno fosse denominado de esforço, e seu causador de *stress*. Mas, devido à popularidade do termo *stress* na comunidade científica e médica, assim como entre os leigos, decidiu mantê-lo. Propôs a criação do termo *stressor* para designar o agente causador da resposta de *stress* (PAKÁC & PALKOVITS, 2001). Observou também que os organismos, nesta situação, apresentavam involução do timo, aumento da adrenal e ulceração péptica, os quais agrupou como sendo componentes de uma *Tríade Patológia* (SELYE, 1946; 1956).

Anterior à Selye, o fisiologista americano Walter Cannon havia definido a "reação de luta-ou-fuga", como o processo de ativação de fatores endócrinos envolvendo o eixo composto pelo sistema nervoso simpático e a medula adrenal, quando animais eram expostos a situações ou fatores ameaçadores de sua homeostase. Cannon (1939) propôs ainda que o sistema nervoso simpático e a glândula adrenal funcionariam como uma unidade. A reação por ele descrita correspondia à reação de *stress*, mas ele jamais utilizou este termo.

Para caracterizar a tríade Patológica desencadeada pelo *stress*, Selye descreveu a Síndrome Geral de Adaptação (SGA), dividindo-a em três fases. Na primeira fase, de alarme, mecanismos orgânicos de defesa são acionados; tais como a ativação do sistema nervoso simpático (SNS) e da medula adrenal, bem como o eixo hipotálamo-pituitária-córtex adrenal (HPA). Esta fase se caracteriza por aumento das capacidades orgânicas em responder ao estressor, com resposta fisiológica dos órgãos e sistemas à elevação da concentração plasmática das catecolaminas e dos glicocorticóides. A fase seguinte denominada de resistência caracteriza-se pelo equilíbrio, pela volta à homeostase, durante a qual a reação inicial de alarme do estresse continuar-se-ia em mecanismos adaptativos se o estressor fosse mantido ou repetido. Este momento é crítico para o organismo, pois ele está debilitado. Caso a adaptação não seja possível, instala-se a terceira fase, de exaustão. Esta é caracterizada pela

depleção das reservas energéticas e o desenvolvimento de doenças, podendo sobrevir até a morte (SELYE, 1936; 1946; MEERSON, 1984; GRIFFIN, 1989; VAN DER KAR *et al*., 1991; FRANKS, 1994).

Por sua teoria do *stress*, Hans Selye torna-se uma das cem pessoas mais importantes do mundo, como intitula um jornalista francês e a própria literatura, já que seus trabalhos foram citados 362.000 vezes, durante sua vida. O termo *stress* e seus derivados foram incorporados a quase todos os idiomas. Na língua portuguesa, criou-se o termo estresse.

O avanço da ciência não desmerece a teoria de Selye, mas aprofunda seus entendimentos, interpretações e explicita mais o termo. A definição de *stress*: ″resposta não específica do organismo frente a ameaçadores da sua integridade″, foi ampliada. Outros autores defendem que a resposta do organismo frente a diferentes agentes estressores pode aumentar, diminuir ou não alterar a atividade do eixo HPA (GOLDSTEIN, 1995; MUNCK, GUYRE & HOLBROOK, 1984; MASON, 1975), e o seu caráter não específico, é questionado, reconhecendo-se que em nível molecular e celular, a resposta de estresse é específica, pois depende do tipo e da intensidade de agentes estressores.

Neste contexto, diferentes grupos de pesquisadores tentam redefinir estresse e até entendem que novos termos devam ser introduzidos na literatura para melhor exemplificar o que traduziria o fenômeno observado. Sterling & Eyer (1988) sugerem uma nova interpretação do conceito de homeostasia, defendendo a hipótese de que a estabilidade do meio interno é alcançada por meio da mudança e não da constância, e sugerem o termo alostasia para definir este “novo” conceito. Goldstein (2003) adota o novo conceito e escreve: “alostasia tem por essência a regulação ao redor de um estado estável alterado”. Asism sendo, a alostasia dependeria da ativação de processos de adaptação por mediadores químicos, tais como as catecolaminas, os esteróides da adrenal, as citocinas e outros. McEwen & Stellar (1993) criam o termo “carga alostática” para indicar o conjunto dos mecanismos desencadeados com o objetivo de manutenção da alostasia. Se a resposta alostática é suficiente, ocorre adaptação e o organismo é protegido de danos. Propõem também que, se a resposta alostática é prolongada, inadequada, repetida, ou ainda se há falha de adaptação, o resultado é a “sobrecarga alostática”, ou seja, a falha na adaptação e conseqüentes danos a vários órgãos. Nestas condições, estresse é referido como sobrecarga alostática e incapacidade adaptativa (LEVINE, 2005), podendo gerar doenças. Os neurotransmissores, hormônios e citocinas liberados, seriam capazes de manter o estado de alostasia. A permanência contínua ou intermitente e a

intensidade da “nova situação” são componentes que definem o estado de estresse. Novamente, o limiar vem separar o padrão de resposta ao novo. Se a experiência, os conhecimentos anteriores e a dedução das circunstâncias forem similares às vividas, a discrepância entre o que é observado ou vivido e o que é esperado ou programado se reduzirá, eliciando respostas compensatórias que são específicas para cada estímulo e para cada organismo.

Mas, recentemente, DAY (2005) discute com propriedade estes novos conceitos, sugere a retomada da definição original de estresse, e propõem a seguinte redação: “estresse é a resposta do organismo, envolvendo vários sistemas orgânicos às ameaças ou desafios que ultrapassam ou parecem que ultrapassam os mecanismos seletivos“. Assim, o desenvolvimento da Tríade Patogênica descrita por Selye, só aconteceria no momento em que os mecanismos seletivos fossem ultrapassados.

Os agentes estressores são divididos em quatro categorias: físicos, psicológicos, sociais e aqueles que desafiam a homeostase cardiovascular e metabólica. Podem ser agudos ou crônicos, intermitentes ou de exposição contínua, por tempo limitado ou prolongado, diferindo em sua intensidade (PACAK et al., 1998; McCARTY, 1989; van de KAR & BLAIR, 1999)

A resposta de estresse tem caráter adaptativo e visa proteger o organismo e garantir a sua sobrevivência. Neste sentido, o estresse é benéfico quando refere-se a experiências de tempo limitado, que o indivíduo pode superar, e que o levam à sensação excitatória de realização. Por outro lado, o estresse torna-se perigoso para a saúde quando o senso de controle e o domínio são perdidos e estas sensações são frequentemente prolongadas, recorrentes, irritantes, emocionalmente esgotantes e fisicamente esmagadoras ou danosas (McEWEN, 2007). Assim sendo, o estresse teria duas faces: aquela que nos estimula a seguir, desafiar, denominada de *eustresse*, e aquela relacionada às doenças, denominada *distress*. A interpretação do estímulo ser prazeroso ou ameaçador depende de experiências vividas e das expectativas dos resultados. Há agentes estressores que são considerados, na maioria das vezes como negativos, outras dependerão da interpretação individual (Selye, 1974).

A interpretação do agente estressor como ameaçador ou não, e a determinação das respostas comportamental e fisiológica é realizada pelo cérebro. O sistema de estresse envolve áreas corticais e subcorticais, como o sistema límbico, do qual fazem parte a amígdala, relacionada às sensações de medo, e pelo hipocampo, responsável pela memória das informações previamente aprendidas. Participam também, o tronco cerebral, relacionado a

ansiedade, além de outras áreas responsáveis pelas tomadas de decisões (SAPOLSKY et al., 2000; McEWEN, 2007). Porém dois eixos são fundamentais para a resposta do estresse: o neuronal, representado pelo sistema nervoso simpático e medula adrenal (SNSMA), o qual se inicia no tronco cerebral; e o hormonal, representado pelo eixo hipotálamo-pituitária-córtex adrenal (HPA). Estes conectam a interpretação da resposta das regiões centrais com os sistemas periféricos. A ativação do eixo neural resulta na síntese e liberação das catecolaminas. Com a ativação do eixo hormonal, há aumento da síntese e liberação dos hormônios do córtex adrenal, principalmente dos glicocorticóides.

Entre os glicocorticóides, o cortisol é o mais expressivo em humanos, e por isso o mais estudado. Durante o estresse, os glicocorticóides liberados atingem suas células-alvo, incluindo as células cerebrais. A ação central dos glicocorticóides está associada com mudanças comportamentais, neuroquímicas e neurodegenerativas. Alterações neurodegenerativas ocorrem em neurônios do hipocampo. Esta ação explicaria a relação que existe entre o estresse e os glicocorticóides e a interrupção na formação da memória, bem como com a atrofia de neurônios do hipocampo e suas ramificações. A neurogênese que ocorre no hipocampo pode ser inibida pelo estresse e pelos glicocorticóides. Por outro lado, os glicocorticóides também desencadeiam respostas que são neuroprotetoras durante o estresse, aumentando a expressão de marcadores de mecanismos adaptativos (SAPOLSKY et al., 2000). O papel dos glicocorticóides é relevante do começo ao fim da resposta de estresse, e ainda, indiretamente, continua após o desaparecimento do agente estressor. Segundo Sapolsky et al. (2000), os glicocorticóides permitem, estimulam, suprimem o avanço da resposta de estresse e preparam o organismo para estressores subsequentes.

Há receptores de glicocorticóides também na amígdala e no córtex pré-frontal, porém estas regiões ainda são pouco estudadas. Estresse repetido ou crônico por imobilização resultou em surgimento de pequenos dendritos no córtex pré-frontal medial e crescimento dendrítico na amígdala, bem como no córtex orbifrontal (LISTON et al., 2006). O estresse crônico aumenta o medo do desconhecido e o medo condicionado dependente da amígdala (CONRAD et al., 2003). Outro reflexo da hiperatividade da amígdala no estresse crônico é o aumento da agressividade de animais que convivem na mesma gaiola (WOOD et al., 2003). Há uma interação recíproca entre estas áreas cerebrais. O processo da memória emocional com informações contextuais requer interações entre o hipocampo, amígdala e o córtex pré-frontal,

pois este atua inibindo o medo, através da sua ação sobre a amígdala (MILAD & QUIRK, 2002; MORGAN & LeDOUX, 1995).

A capacidade de conviver com o agente estressor, caso não seja possível eliminá-lo, é definida por alguns autores, como resiliência e os mecanismos acionados para obtê-la constituem as estratégias de convivência (ou *coping*). A percepção do agente estressor pode ser reduzida ou diminuída quando este proporciona uma resposta positiva (ERIKSEN & URSIN, 2004). Estilos de enfrentamento (*coping styles*) aos agentes estressores podem ser baseados em experiências ou recompensas, principalmente aquelas que resultaram em sucesso (FOLKMAN & LAZARUS, 1990).

Vários são os estilos de *coping* além de estilos pessoais. Entretanto, a grande desigualdade social na manifestação de queixas e doenças pode estar relacionada à motivação para escolha do estilo de vida. Esta pode representar padrões desiguais de reforço para o desenvolvimento de *coping* (ERIKSEN & URSIN, 2002).

Marcadores da resposta de estresse

Os hormônios sintetizados e liberados pelo córtex adrenal, bem como as catecolaminas, sintetizadas e liberadas pelos neurônios simpáticos e pela medula adrenal são considerados marcadores da resposta de estresse. Determinar suas concentrações plasmáticas exige o emprego de técnica invasiva via punção venosa, de modo que interpretar seus resultados exige cautela. Além disso, esta técnica permite apenas a informação de um valor pontual, em um determinado momento do dia, e a quantificação das concentrações das catecolaminas é feita por cromatografia líquida de alto desempenho, que apresenta considerável dificuldade técnica e alto custo. Por outro lado, os hormônios esteróides do córtex adrenal podem ser detectados por meio de técnicas mais simples.

Assim sendo, a determinação dos biomarcadores de estresse na saliva é um método alternativo que apresenta vantagens, pois não é invasivo, não causa estresse e pode ser feito pela própria pessoa, em qualquer lugar. Por ser inócua e indolor, favorece a colaboração de voluntários e permite a coleta de material em diversas situações do cotidiano. A concentração salivar é diretamente proporcional à concentração da fração livre, biologicamente ativa, do hormônio no sangue (VINING & MCGINLEY, 1987), e é independente do fluxo salivar (KIRSCHBAUM & HELLHAMMER, 1994; DUCLOS et al., 1998). Somado a isso, podem ser feitas coletas seriadas de amostras que permitem determinar, além da concentração

instantânea, o ritmo de secreção do hormônio ao longo de períodos (dia, noite, ou outros diferentes períodos), bem como sua variação durante fases distintas e/ou críticas da vida de qualquer população a ser estudada (KIRSCHBAUM & HELLHAMMER, 1994).

Nossa colaboração

O grupo de pesquisa do qual fazemos parte tem uma vasta e enraizada experiência nos estudos dos mecanismos biológicos do estresse e na determinação da concentração sanguínea ou salivar dos hormônios esteróides como biomarcadores do estresse e da recuperação, principalmente o cortisol e a testosterona. Estes foram determinados em estudantes durante o ano de preparo para o exame vestibular, em atletas jogadores de futebol, basquetebol e nadadores durante fases de treinamento e de competições, em jovens executivos também durante suas rotinas de trabalho ou dias de repouso, em indivíduos de baixo *status* socioeconômico (GARCIA et al., 2008), e em portadoras de endometriose (PETRELLUZZI et al., 2006). O cortisol também foi utilizado como marcador biológico do índice de estresse em mães cuidadoras de crianças portadoras de paralisia cerebral (BELLA et al., 2008).

Os estudos com estresse em humanos foram iniciados no Laboratório de Estudo do Estresse (LABEEST) em 2001, quando trabalhamos com jogadores juniores de futebol de uma equipe de Campinas. Acompanhamos duas temporadas esportivas consecutivas que ocorreram de março à janeiro. Naqueles atletas determinamos concentração sérica de cortisol e de testosterona, correlacionando-as com o condicionamento físico e com os resultados obtidos nos jogos (GARCIA et al., 2008).

O pressuposto de que, sendo o Brasil considerado “o país do futebol” facilitaria o encontro de voluntários, não se mostrou verdadeiro. Não obstante, a equipe enfrentou os mais diversos obstáculos ao iniciar este trabalho: as freqüentes alterações na equipe técnica, que resultavam em dificuldades para dar andamento ao protocolo de pesquisa, dificuldades com a implantação da metodologia, resistência das equipes técnicas em avaliar regularmente o desempenho físico dos atletas, além do conturbado calendário futebolístico brasileiro, dificultaram a realização da pesquisa científica e, a nosso ver, contribuem para o empirismo do trabalho nesta modalidade esportiva.

Outro fator limitante foi a coleta de sangue, que *per se* é um agente estressor para a maioria das pessoas, especialmente para atletas em momentos de competições. Para superar esta dificuldade implantamos a técnica de coleta da saliva e determinação da concentração

salivar de cortisol como indicativo do índice de estresse. Esta técnica nos permitiu avaliar momentos que seriam impossíveis de serem analisados por meio da tradicional coleta de sangue. Agora podemos fazer seqüências seriadas em dias específicos, que permitem determinar o ritmo circadiano de cortisol e a área sob a curva, bem como a resposta do cortisol ao acordar, além de realizar coletas em dias de competição, como também minutos antes e após cada prova ou jogo.

Não obstante, questões pertinentes à técnica da coleta surgiram. O processo seria através de um tubo coletor? O fluxo salivar alteraria o resultado? Haveria a necessidade de um meio de transporte especial? As amostras deveriam ser mantidas em baixa temperatura?

A literatura existente àquela época apontava o método de determinação da concentração salivar de cortisol como sendo de fácil execução, simples de coletar, indolor e sem maiores complicações para o procedimento. O cortisol é estável à temperatura ambiente, o que diminuía as exigências quanto à temperatura de conservação e transporte das amostras, além de sua concentração na saliva ser independente do fluxo salivar (KIRSCHBAUM & HELLHAMMER, 1994). Ficou definido que o transporte até o laboratório poderia ser feito em caixas com gelo ou até em temperatura ambiente sem perigo de degradação das amostras, e que estas podem ser conservadas em geladeira e/ou freezer domésticos até o envio ao laboratório.

A utilização de salivette®, facilitando a coleta pelo voluntário foi a alternativa com o melhor grau de viabilidade e eficácia. A seleção do material a ser utilizado privilegiou *kits* diagnósticos com características de boa especificidade e sensibilidade.

Após os primeiros ensaios detectamos que a presença de muco nas amostras prejudicava as análises, o que obrigou uma alteração no protocolo de centrifugação. Atualmente o procedimento orienta a centrifugação durante 20 minutos, a 2800 rpm e a uma temperatura de 4°C. Isto resultou em maior precisão na pipetagem, redução na diferença entre as duplicatas e na variação intra e inter-ensaio.

Após a centrifugação, a distribuição de pequenos volumes da amostra total em microtubos evita o descongelar e recongelar de amostras e garante a possibilidade de repetição do ensaio, quando isto se faz necessário, utilizando amostras intactas.

Com a melhora dos protocolos de coleta e análise, e o aumento da confiança no método, problemas com a aderência aos horários do protocolo de coletas começaram a ser percebidos, como bem corrobora a literatura (KUDIELKA et al., 2003; THORN et al., 2006;

LASIKIEWICZ et al., 2008). A partir de então, todos os cuidados passaram a ser tomados para que o voluntário tenha total consciência da importância da sua colaboração, da importância da pesquisa que está sendo realizada, e de que em caso de dúvida, possa entrar em contato com os membros da equipe de pesquisa. A presença do pesquisador responsável junto dos voluntários se mostrou um aspecto bastante importante no sentido de aumentar a adesão ao protocolo, entre outros aspectos. Para tanto, foi implantados no laboratório um protocolo de controle rígido de acompanhamento das pesquisas com coleta de saliva em seres humanos.

A partir desta trajetória, determinamos o índice de estresse em jogadores de futebol durante duas temporadas esportivas consecutivas, em estudantes durante o ano de preparo para o vestibular, em atletas de basquetebol também duas temporadas esportivas e em cidadãos brasileiros pertencentes a duas classes sócio-econômicas distintas. Os resultados obtidos foram organizados em manuscritos, os quais serão aqui apresentados.

OBJETIVO

Foram objetivos deste trabalho: determinar a concentração salivar de cortisol como indicador dos índices de estresse em:

1- atletas jogadores de futebol e de basquetebol durante temporadas esportivas e correlacionar estes índices com desempenho físico e desempenho nos jogos;

2- estudantes durante o ano de preparo para o vestibular;

3- indivíduos de baixo status sócio-econômico;

4- atletas de basquetebol durante temporada esportiva e correlacionar este índice com resultados obtidos nos jogos: vitória ou derrota.

PRIMEIRO MANUSCRITO

CORTISOL AND TESTOSTERONE SERIC CONCENTRATIONS IN YOUNG SOCCER PLAYERS

CORTISOL AND TESTOSTERONE SERIC CONCENTRATIONS IN YOUNG SOCCER PLAYERS

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Running title: cortisol and testosterone levels in soccer players

Abstract

The aim of this study was to evaluate the basal serum concentrations of cortisol and testosterone, as reliable markers of stress and recovery, respectively, in young soccer players of a first ranking Brazilian team. The hormone concentrations were assessed 7 times during two training seasons, immediately before and after two consecutive tournaments. Cortisol serum concentrations were maintained near the maximum of the normal range (467.4 ± 30.6 $nmol.L^{-1}$), increased by the end of the season (692.4 ± 41.5 $nmol.L^{-1}$), and returned to the basal levels after the playoffs (565.5 ± 46.8 $nmol.L^{-1}$). Testosterone concentrations remained stable throughout the entire period, although near the lower normal limit, independent of athletes' position. These results indicate that during this period, the team experienced a predominant catabolic state, which might have contributed to the decrease in body fat percentage observed and in the 12-min run test, mostly in the midfielders. We conclude that intensive training and sequence of tournaments with no intervening resting periods contributed to stress. Therefore, sport practice schedules need to be more carefully organized, in order to decrease its impact on player's physiology. Constant monitoring, with the determination of hormonal levels should be included in routine evaluations of soccer players.

Keywords: soccer, cortisol, testosterone, stress

Introduction

Soccer involves high-intensity, intermittent exercise relying predominantly on aerobic energy pathways combined with intermittent anaerobic incidents (Kinkerdall, 1985; Ekblom, 1986; Bangsbo, 1994). That is why conditioning programs for soccer players must focus on aerobic capacity, strength, power, speed, and speed endurance. Appropriate conditioning must also focuses on the maintenance or improvement of standards of performance, associated with the ability of body systems to recover and regenerate following strenuous training sessions and games, as well as the ability to cope with the psychological stress related to tournaments (Carli, Di Prisco, Martelli, & Vitti, 1982; Ekblom, 1986; Kraemer, French, Paxton, Hakkinen, Volek, Sebastianelli et al., 2004). Therefore, the training programs and competitions are constantly challenging the athlete's body's homeostasis, thus triggering the stress reaction, which may lead to adaptation in higher levels of performance or reduced performance and overtraining.

Stress has been defined as the body multi-system response to any challenge that overwhelms, or is judge to overwhelm, selective homeostatic mechanisms (Day, 2005). Although the stress response involves many organic systems, cortisol has been identified as a reliable marker of stress. On the other hand, testosterone has been pointed out as an indicator of explosive performance (Cardinale & Stone, 2006) and recovery, and the balance between cortisol and testosterone has been proposed as indicating predominance of catabolism or anabolism during pre-season training and competition (Kraemer, Patton, Gordon, Harman, Deschenes, Reynolds et al., 1995; Kraemer, 2000). Such balance might be influenced by playing out of or at home (Neave & Wolfson, 2003), the outcomes (Suay, Salvador, Gonzalez-Bono, Sanchís, Martinez, Martinez-Sanches,et al., 1999; Edwards, Wetzel & Wyner, 2006) and if the player is a starter or a replacer (Kraemer et al., 2004).

However, the data in the literature related to this issue are not consistent, especially for team sports such as soccer (Banfi & Dolci, 2006). It has been demonstrated that during the course of a competitive soccer season, basal levels of both cortisol and testosterone can become significantly higher than those found during the pre-season period (Carli et al., 1982). Other studies have shown increases in the resting serum concentration of testosterone (Staron, Karapondo, Kraemer, Fry, Gordon, Falkel et al., 1994) and decreases in that of cortisol (Staron et al., 1994; Kraemer et al., 1995) during long term, normal strength training programs. On the other hand, those alterations were claimed to occur only in starting players but not in non-starters (Gorostiaga, Izquerdo, Ruesta, Iribarren, Gonzalez-Badillo & Ibánez , 2004).

An additional component of the stress of soccer involves the calendar of matches which frequently deprives players of resting periods between sequences of competitions, so that the players may enter the competitive season in a predominantly catabolic state which may negatively influence the performance and, then, outcomes.

In the present study, the serum concentrations of cortisol and testosterone were evaluated as markers of the catabolic/anabolic balance in young soccer players, during two training periods, before and after two consecutive tournaments. The aim was to evaluate the interference of the training program and sequence of tournaments in the performance and in the basal concentrations of these hormones as markers of the players' metabolic state.

Methods

Subjects

Thirty male soccer players (16 to 19 years old) of a soccer team which was classified in the top rank league in the State of São Paulo, Brazil (Associação Atlética Ponte Preta, Campinas, SP, Brazil) participated in this study. The research design was approved by the Committee for Ethics in Human Research of the Medical School, of the State University of Campinas, and it was explained to the players, their parents and the team coaches. The players volunteered for the study, and they and their parents signed the written informed consent forms. Before engaging in the study, players were submitted to a clinical examination by the team physician in order to verify their health conditions, height and body mass. Those who reported health problems or who were taking any medication were excluded from the study. Body mass and clinical conditions were again evaluated at the end of the training season. At the conclusion of the study, all the subjects met the predetermined criteria and had completed the season without serious injury, except for one athlete who had a shoulder injury with indication for surgery.

Conditioning program and tournament features

The training season started in March. The training program macro cycle consisted of three consecutive phases respectively focused in strength, power and velocity lasts from March to July, before the first tournament, that occurred in August and September. The second macro

cycle lasts from October till December, following the same program, although for a shorter period. The tournament occurred in January. During the training season, players trained 20 h a week distributed in 10-12 periods. Both tournaments consisted of two phases. In the first phase of the tournament, all teams played ten games. Those which won more games were classified for the second phase. The team that we evaluated won only two of ten games and it was eliminated. The second tournament first phase consisted of three games. The team won only one and it was again out of the tournament after the first phase. All games were played out of home and the team participants in 2 or 3 games a week. In the days with no games, throughout the tracking, the players continued to participate in regular team practice (for speed, agility, and endurance, as well as in simulated games). All workouts were supervised by the team coaches.

Experimental approach

Monitoring started in June, after three months of training, and continued for 26 weeks, until the following January. This period included two training periods and two tournaments, one occurring in August and September, and the other in January. Measurements of physical performance were made once in June (after the power phase of the first training season, T1) and again in November (during the power phase of the second training season, T5); serum concentration of cortisol and testosterone were assessed seven times during this period: in June (T1) and July (immediately before the first tournament, T2); in October (4 days after the end of the tournament and at the beginning of the second training period, T3); in November and December (after the strength phase of the second training season, T4 and after the power phase of the second training season, T5, respectively); and two times in January (immediately before and after the second tournament, T6 and T7, respectively). A minimum interval of 15 days separated evaluations.

Therefore, T1 and T5; T2 and T6; T3 and T7 were correspondent phases for the first and second seasons; before and after the first and the second tournaments, respectively.

Physical performance testing

Physical performance was tested on a previously determined morning in the club gymnasium. All the players were familiar with the environment and the tests, which were conducted under the supervision of the team coaches and the investigators.

Following instructions, the subjects performed three continuous maximal effort contractions (extension/flexion) throughout the complete range of motion of the leg. The greatest strength produced during each of the assessments was recorded, and the data were used to represent the maximal isokinetic strength of knee for that velocity.

Aerobic capacity was evaluated using the "12-minute run test" in which the athlete must run at least 2700 m in 12 min. These two tests must be performed by each athlete before being included in an official competition, as ruled by the "Federation Internationale de Football Association" (FIFA) and the "Union of European Football Association (UEFA)".

Blood sample collection and analysis

Blood samples were collected by a qualified nurse in the club infirmary, between 8:00 and 10:00 am, under the supervision of the investigators, always on Mondays, since the athletes did not train on Sundays and Saturdays. The subjects had fasted for 12-h prior to the tests. There was no control on their activities did on the weekend. A 10-ml blood sample was obtained from the antecubital vein of each soccer player. Blood samples were allowed to clot at room temperature, and centrifuged at 2500 rpm, for 10 minutes, at $4^{o}C$. The serum was them removed and stored at $-80^{o}C$ until subsequent analysis. Serum concentrations of testosterone and cortisol were determined in duplicate using a commercial immunoassay kit (Diagnostic System Laboratories Inc., Webster, TX, USA). The intra- and inter-assay variances for cortisol were 5 and 8%, respectively, and for testosterone, 6 and 4 %, respectively.

Statistical analyses

Data are presented as means ± s.e.m. Student t test, one-way analysis of variance or Kruskall-Wallis test were used for comparison between periods. When a significant F value was obtained, the Tukey or Dunn tests were used to locate the differences between means. The level of significance was set at $p<0.05$.

Results

The soccer players participating in this study were 18.3 ± 0.1 years old and 1.78 ± 0.01 m height. Although the mean players body mass showed no significant difference between

June and November (for the first and second training seasons, respectively), there was a slight but significant decrease in the mean percentage of body fat during this period (Table 1).

Table 1. Body mass and percent of body fat in young soccer players in June (T1, during the first training season) and in November (T4, during the second training period)

	Body mass (Kg)		% of body fat	
Positions	T1	T4	T1	T4
All team	73.58 ± 1.13 (30)	72.78 ± 1.01 (34)	12.18 ± 0.40 (30)	11.13 ± 0.31 (34)*
Midfielders	70.59 ± 1.15 (17)	71.70 ±1.07 (21)	11.91 ± 0.57 (17)	10.84 ± 0.38 (21)*
Defenders	76.50 ± 3.95 (4)	79.45 ± 1.16 (4)	13.55 ± 0.63 (4)	12.69 ± 0.28 (4)
Attackers	76.56 ± 2.61 (8)	72.31 ± 2.82 (8)	12.16 ± 0.84 (8)	11.03 ± 0.80 (8)
Goalkeeper	71.7 (1)	72.6 (1)	11.54 (1)	11.80 (1)

Data are presented as means ± s.e.m. of (n) subjects. * Statistically significant difference; Student's *t* test for all team and Kruskal-Wallis test followed by the Dunn test for the other comparisons ($p<0.05$).

When the players are grouped by position, it is possible to see that all the players had a decrease in the percentage of body fat; however, the difference was statistically significant only for the midfielders. A significant change was observed in the performance indicators for this period in the midfielders who presented a decrease in the distance run during the 12-min run test. The goalkeeper also presented a decrease in the knee extension strength and in the distance run during the 12-min run test (Table 2).

Table 2. Maximal effort in knee contraction (extension/flexion) throughout the complete range of motion of the leg and aerobic capacity (analyzed with a 12-minute run test) of young soccer players in June (during the first training season) and in November (after the end of the tournament and during the second training period)

	June (T1)	November (T4)
Knee extension strength (Kg)	107.00 ± 3.17 (24)	102.70 ± 3.03 (23)
Midfielders	98.88 ± 4.7 (13)	97.63 ± 3.3 (12)
Defenders	119.0 ± 9.7 (4)	121.2 ± 15.0 (3)
Attackers	111.0 ± 4.4 (5)	107.5 ± 3.4 (7)
Goalkeeper	128 (1)	80 (1)
Knee flexion strength (Kg)	65.37 ± 1.47 (26)	66.21 ± 1.38 (22)
Midfielders	62.9 ± 1.2 (17)	64.0 ± 1.3 (13)
Defenders	72.0 ± 6.5 (4)	78.0 ± 5.3 (3)
Attackers	67.5 ± 4.5 (4)	67.4 ± 1.6 (5)
Goalkeeper	60 (1)	60 (1)
12-minute run test (m)	2958 ± 27 (23)	2824 ± 47 (25)*
Midfielders	2967 ± 31.4 (15)	2896 ± 38.1 (14)*
Defenders	2923 ± 99.4 (4)	2923 ± 78.6 (4)
Attackers	2953 ± 46.7 (3)	2790 ± 72.3 (6)
Goalkeeper	2780 (1)	2640 (1)

Data are presented as means ± s.e.m. of (n) subjects. * Statistically significant differences between performance in June and November (Student's *t* test for all team and Kruskal-Wallis test followed by the Dunn test for the other comparisons; $p<0.05$).

The serum concentrations of cortisol and testosterone were assessed seven times during the six months period of the study. The cortisol serum concentrations remained stable throughout the first season (T1, 467.4 ± 30.6 $nmol.L^{-1}$; T2, 573.3 ± 42.3 $nmol.L^{-1}$; T3, 605.4 ± 55.7 $nmol.L^{-1}$) and during the conditioning season (T4, 579.5 ± 49.7 $nmol.L^{-}1$). In the end of the second conditioning season (662.5 ± 55.8 $nmol.L^{-1}$, T5) and immediately prior to the second tournament (692.4 ± 41.5 $nmol.L^{-1}$, T6) there were a significant increase in the serum

concentration of cortisol, although recovery took place after the playoffs (565.5 ± 46.9 nmol.L^{-1}, T7), as shown in Figure 1.

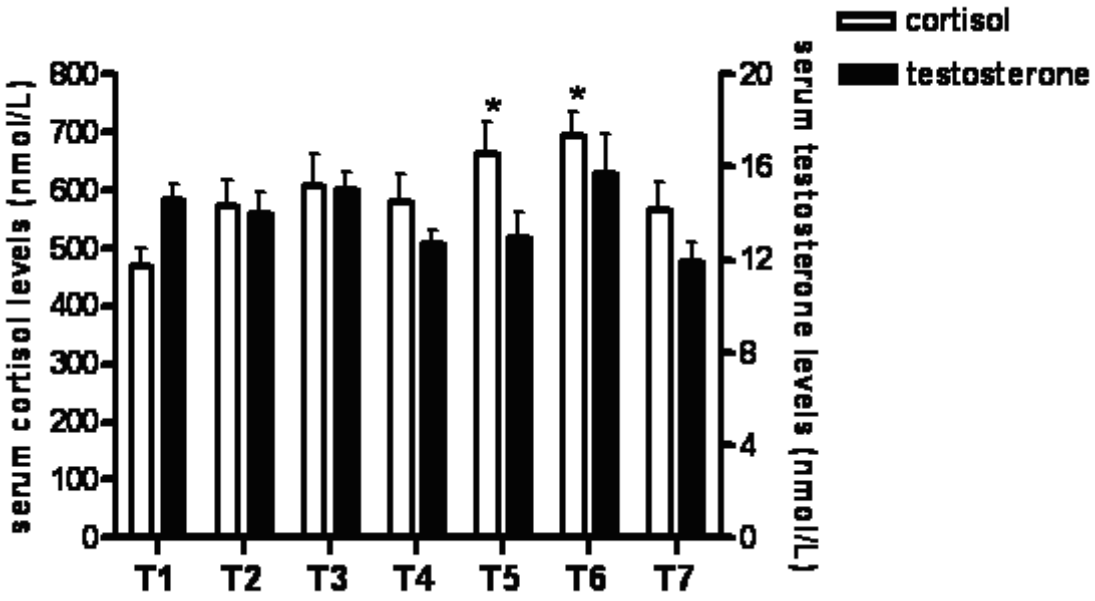

Figure 1. Serum concentrations (nmol/L) of cortisol and testosterone in young soccer players as assessed 7 times during a 26-week period, including two training periods (T1, T2, T4 and T5) and two tournaments (T3, T6 and T7). Vertical bars represent the means ± s.e.m. of the number of subjects indicated in Tables 3 and 4. * Statistically significant difference in relation to T1 (ANOVA for repeated measures plus Tukey test, $p<0.05$).

The serum concentration of testosterone remained stable during the entire period analyzed (minimum of 11.91 ± 0.85 at T7 and maximum of 15.68 ± 1.74 nmol.L^{-1} at T6). As a consequence, the testosterone/cortisol ratio showed a significant difference between T1 and T5 (Figure 2). In November, when the players were starting the power training period of the year second conditioning season, there was a significant positive correlation between the T/C ratio and the percentage of body fat mass ($r = 0.57$; $p<0.05$). This difference was more pronounced and statistically significant among the midfielders. The differences between other two related periods were not statistically significant (T2 and T6; or T3 and T7).

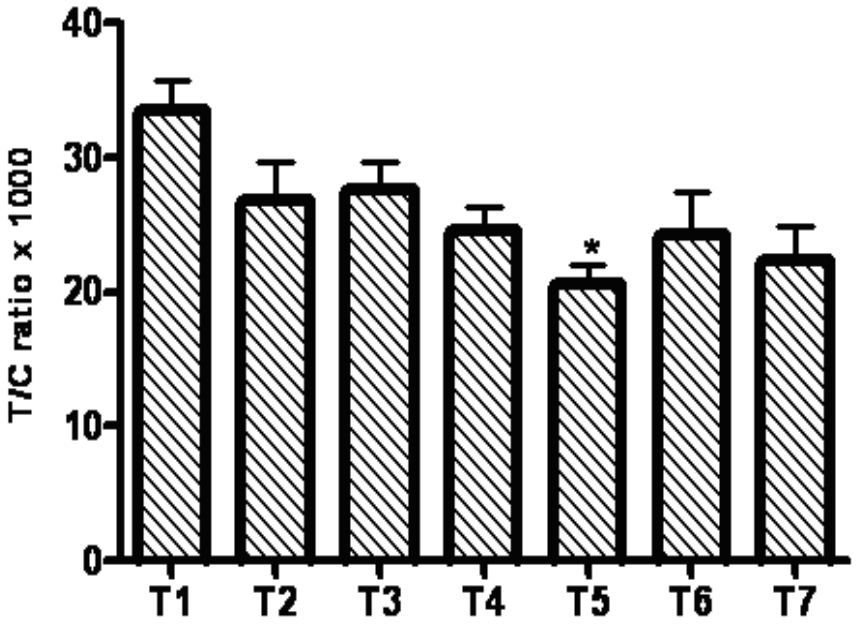

Figure 2. Testosterone:cortisol ratio in young soccer players as assessed 7 times during a 26-week period including two periods of training (T1, T2, T4 and T5) and two tournaments (T3, T6 and T7). Vertical bars represent the means ± s.e.m. of the number of subjects indicated in Tables 3 and 4. * Statistically significant difference in relation to T1 (ANOVA for repeated measures plus Tukey test, $p<0.05$).

When the players were grouped by the position that they occupied at the team (Figure 3A), it is possible to see that the serum concentrations of cortisol showed a similar pattern for the midfielders and the goalkeeper, who presented an increase at the beginning of the first conditioning season (T2 vs. T1), followed by a decrease and then by another increase at the beginning of the second tournament (T5 vs. T4). During all the second season of conditioning and tournament the serum cortisol concentrations remained higher than during the first season. The goalkeeper serum cortisol concentration was kept in a lower level than the midfielders during all the period. On the other hand, the attackers' pattern is the opposite concerning to the serum cortisol concentrations, since they presented a very high serum level of cortisol at T1, then it decreased at the beginning and the end of the tournament season (T2 and T3). During the second training season they again presented an increase in the serum cortisol levels (T4 vs. T3) followed by a decrease during the tournament (T6 and T7 vs. T4). The defenders presented an increase in the beginning of the first training season and then a decrease until T5, followed by an increase only at T6. Nevertheless, the statistical comparison between attackers

and defenders was not possible because of the small number of subjects in those positions (Table 3).

Table 3. Cortisol serum concentrations (nmol/L) of soccer players grouped by the position in the team as assessed 7 times during a 26-week period including two periods of training (T1, T2, T4 and T5) and two tournaments (T3, T6 and T7)

	Midfielders	Defenders	Attackers	Goalkeeper
T1	459,9 ± 28.7 (15)	450,8 ± 58.9 (4)	727,1 ± 158.9 (3)	248.4 (1)
T2	593,7 ± 68.7 (13)	628,9 ± 132.4 (3)	516,2 ± 83.6 (5)	--
T3	627,3 ± 69.5 (10)	484,4 ± 75.9 (2)	470,6 ± 50.7 (6)	415.1 (1)
T4	566,4 ± 44.0 (11)	389,9 ± 120.0 (3)	647,9 ± 163.6 (4)	359.1 (1)
T5	661,9 ± 71.1 (10)	267,2 (1)	562,3 ± 66.3 (5)	515.0 (1)
T6	734,0 ± 65.2 (11)*	581,8 ± 57.1 (2)	591,3 ± 78.6 (6)	--
T7	548,3 ± 55.5 (6)	520,7 ± 102.8 (3)	515,7 ± 62.2 (2)	--

Data are presented as means ± s.e.m. of (n) subjects. * Statistically significant difference between T6 and T1 (Kruskal-Wallis test followed by the Dunn test; $p<0.05$).

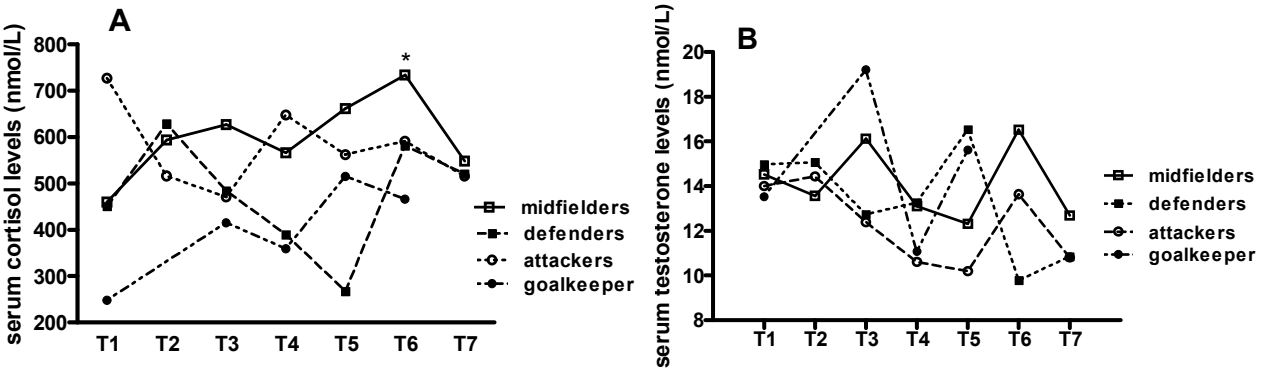

Figure 3. Cortisol (panel A) and testosterone (panel B) serum concentrations of soccer players grouped by the position in the team as assessed 7 times during a 26-week period including two periods of training (T1, T2, T4 and T5) and two tournaments (T3, T6 and T7). The number of subjects, means and s.e.m. values are in Tables 3 and 4. Panel A, * Statistically significant difference between T6 and T1 (Kruskal-Wallis test followed by the Dunn test; $p<0.05$), Panel B There were no statistically significant difference between groups and periods (Kruskal-Wallis test followed by the Dunn test; $p>0.05$).

Moreover, some of the players did not show up in some of the evaluations, reducing even more the number of subjects in each group. Those considerations should be extended to the serum concentrations of testosterone, since no significant alterations were observed in any group (Figure 3B and table 4).

Table 4**.** Testosterone serum concentration (nmol/L) of soccer players grouped by the position in the team as assessed 7 times during a 26-week period including two periods of training (T1, T2, T4 and T5) and two tournaments (T3, T6 and T7)

	Midfielders	Defenders	Attackers	Goalkeeper
T1	14.53 ± 1.2 (15)	14.97 ± 1.1 (4)	14.00 ±1.5 (3)	13.53 (1)
T2	13.57 ± 1.1 (12)	15.07 ± 1.9 (3)	14.43 ± 3.9(5)	--
T3	16.13 ± 1.2 (11)	12.74± 1.3 (3)	12.38 ± 0.9 (6)	19.21 (1)
T4	13.10 ± 1.0 (11)	13.26 ± 2.3 (3)	10.61 ± 0.9 (5)	11.08 (1)
T5	12.31 ± 1.2 (10)	9.20 ± 0.0 (2)	10.20 ± 1.4 (5)	15.62 (1)
T6	16.52 ± 2.4 (11)	9.79 ± 3.1 (2)	13.63 ± 3.7 (6)	--
T7	12.69 ± 1.4 (6)	10.84 ± 1.8 (3)	10.80 ± 2.0 (2)	13.53 (1)

Data are presented as means ± s.e.m. of (n) subjects. There were no statistically significant difference between groups and periods (Kruskal-Wallis test followed by the Dunn test; $p>0.05$).

Discussion

The aim of a conditioning program is to improve athletic performance. The program adopted for Brazilian players tends to be divided in periods during which specific aspects such as strength, power resistance and velocity are worked on. The results presented here have shown that the conditioning program imposed on this team of young soccer players did not

result in any improvement or decrease neither in knee extension and flexion strength nor in performance on the 12-minute run test, except for the midfielders that showed a decrease in the distance run during the test and in the percent of body fat after the end of the two consecutive training periods and the intervening tournament. Filaire, Bernain, Sagnol & Lac (2001) examined adult soccer players during a season of competitions, and reported no alteration in body mass or percentage of body fat for the entire team, although there was an increase in the maximum oxygen consumption at the end of the season. Those authors did not group the athletes by position in the team. The data presented here show that the decrease in the body fat percentage, which was significant for the whole team is due to the midfielders profile. (Table1).

Kraemer et al. (2004) studied the evolution of the physical performance of young soccer players during an 11-week competitive season and compared the results of the starters and non-starters. These authors demonstrated a decrease in isokinetic leg strength and in the peak torque of knee extensors, but no changes in the peak torque of knee flexors or sprint time. Starters also revealed a significant decrease in vertical jump performance not shared by non-starters. Those authors observed that all the players presented a decrease in performance, especially at the end of the season, a finding suggesting that performance adaptations may be independent of total match play (Kraemer et al., 2004). We did not monitor total match play and did not evaluated starters or non-starters apart.

Whereas cortisol have been identified as a reliable marker of catabolism and training stress, the presence of testosterone leading to anabolism has been suggested to be an indication of recovery (Aldercreutz, Harkonen, Kuoppasalmi, Naveri, Huhtaniemi, Tikkanen, et al., 1986; Hakkinen, Pakarinen, Alen, & Komi, 1987; Passelergue, & Lac, 1999; Kraemer et al., 2004). The data reported here have shown that testosterone serum concentrations did not change during the analyzed period and that these concentrations were near to the lower limit, although within the normal range, which is between 10.4 and 41.6 $nmol.L^{-1}$. These data suggest that the anabolic processes have been kept to a low level throughout the entire season. Moreover, considering that testosterone is important to explosive performance (Cardinale & Stone, 2006), those low levels might have contributed to the poor results obtained by this team, i.e., it was eliminated in the first phase of both tournaments.

Reports on changes in resting testosterone concentrations during resistance training have been inconsistent (Alen, Pakarinenm Hakkinen, et al., 1988; Potteiger, Judge, Cerny, et

al., 1995; Kraemer & Ratamess, 2005). Substantial changes in volume and intensity may elicit transient changes in resting testosterone concentrations; however, values may return to baseline when the individual returns to 'normal' training (Kraemer & Ratamess, 2005).

On the other hand, in the team analyzed here, cortisol serum concentrations were maintained, in the entire period, near the maximum value of the normal concentration range which is between 110.4 and 745.2 $nmol.L^{-1}$. The serum concentrations of cortisol were higher at T5 and T6, indicating that the team had suffered more stress during the second than the first training season. At the end of tournament (T7), serum concentrations of cortisol had returned to values similar to those observed at T1. These results indicate that throughout the period, the team had experienced predominantly a catabolic state. This might have contributed to the decrease in the percentage of body fat observed mostly in the midfielders that presented a significant increase in the serum cortisol concentrations at T6 and reduction in the fat percentage as well as a decreased performance in the 12-min run test. The testosterone/cortisol ratio also decreased significantly at T5, mostly due to an increase in the cortisol. At this time (T5), there is a positive correlation between T/C ratio and body fat mass percentage. The T/C ratio has been suggested to be an indirect measure of the anabolic/catabolic properties of the skeletal muscle (Fry & Kraemer, 1997). Some studies have previously shown changes in the T/C ratio during strength and power training, which were positively related to performance improvements, whereas others have shown no changes (for a review, see Kraemer & Ratamess, 2005).

The basal concentrations of cortisol and testosterone reported here are quite similar to those reported by Kraemer et al. (2004). However, those authors observed an increase in the circulating testosterone levels at the end of the season, signaling a reduction in total stress which revealed a situation contrasting with what was observed in the team analyzed here. These changes may be related to a difference in the approach to training of the team, and may have contributed to team performance during tournaments. Whereas Kraemer's athletes trained about 10 h/week, the athletes that we analyzed used to train 20 h/week. Probably, a large period of rest between the training sessions is important to allow the increase on testosterone serum concentrations and then to lead to anabolism and recovery in a higher level, what was not done in the team that we analyzed. Whether the changes in hormonal profile are a consequence of the soccer training approach, some alternative strategies should be devised to reduce cortisol serum concentration or enhance testosterone concentrations immediately prior

to tournaments. This would change the prevailing catabolism into an anabolic state that will contribute to a better performance during games. The increase in testosterone concentration observed by Kraemer et al. (2004), not observed in the present study, may represent a rebound of physiological functioning with the reduction of stress, possibly related to the team's performance. Florini (1989) has reported the antagonistic relationship between anabolic and catabolic hormones, and indicated that a decrease in plasma testosterone coupled with a catabolic state accompanying elevated cortisol levels can result in a reduced physical performance. Testosterone levels were unaltered in the team that we analyzed; however, there was an increase in the already elevated concentration of cortisol, although this did not surpass the level of normality. This change may have influenced the strength of the catabolic state, contributing to the poor results obtained by the team.

As previously emphasized by Kraemer et al. (2004) and also shown in the present study, the soccer players entered the tournaments in a catabolic state; whether this metabolic condition is reverted during the tournament (as occurred in the study of Kraemer and his colleagues) or not (as occurred in the present study) may determine the performance of the team. Therefore, it can be speculated that some strategies to increase testosterone serum concentration or decrease cortisol concentration might lead to better results. The data presented here reinforce the hypothesis of Kraemer et al. (2004) that intensive training both during practice and tournaments contributes to chronically elevated cortisol concentrations and low concentrations of testosterone. An additional source of stress in the case of the team analyzed in the present study was the sequence of tournaments with no intervening resting periods for the athletes, contributing even more to the catabolic state. These results suggest that sport practice schedules may need to be more carefully organized by the heads of soccer federations, who may not appreciate the impact of decisions about training on the physiological status of the players. In this context, constant monitoring, including the evaluation of hormonal levels of the athletes during conditioning and tournaments, should be considered as part of the training program and included in routine evaluations of soccer players.

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SEGUNDO MANUSCRITO

SALIVARY CORTISOL LEVELS IN BASKETBALL ATHLETES

SALIVARY CORTISOL LEVELS IN BASKETBALL ATHLETES

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Running title: salivary cortisol levels in basketball players

ABSTRACT

Aim: To quantify the salivary cortisol levels in basketball athletes in the days of games during a competitive season and to investigate if there is any correlation between salivary cortisol levels before and after the games with the game result.

Method: 10 male basketball athletes, 19-27 year-old, of a local club volunteer to participate in this study after approval of the head coaches and the Institutional Ethics Committee for Research (UNICAMP). Saliva samples were collected immediately after wake up, 30 min later, before lunch, before dinner and before going to sleep, in salivettes®, in games days. The competitive season was composed by 18 games occurring on Wednesdays or Saturdays and Sundays from March till May. Games 1 and 10 were played at 11 am, games 8, 12 and 16 at 4 pm, and the other occurred at 8 pm. Saliva samples were also collected before and after each game. The samples were frozen until analysis using a commercial kit (Diagnostics System Laboratories (DSL), USA). Data were compared by ANOVA plus Tukey test and Pearson's correlation test. Differences were considered significant when $P<0.05$.

Results: Basal salivary cortisol levels were higher in the days of the first and second games compared to the other ones. In most of the game days, the cortisol circadian rhythm has been preserved. There was an anticipatory increase in the salivary cortisol levels before each game that were even higher at the end of the games. There was a positive correlation between the values before and after the game ($r = 0.85$; $P=0.0001$). In the games played in the morning there was a correlation between the intensity of cortisol levels increase and victory, and this is not seen in the games played in the afternoon or at night. There was no correlation between salivary cortisol levels and the game results or the game being played at home or not. The awakening cortisol responses were lower than expected.

Conclusion: In the beginning of the competitive season athletes are exposed to higher levels of cortisol. However, the cortisol circadian rhythm has been preserved during all the season. In the morning games the increase of cortisol levels are related to the game result, with higher increases correlated with victory. This does not happen for the afternoon and evening games. There was not adherence to the protocol of samples collection mostly immediately waking up and 30 min after.

Key words: cortisol, stress, sports, competition, basketball

Introduction

Sports practice activates the stress system (Charmandari et al., 2005) causing the increase of the stress hormones plasma levels, such as cortisol and catecholamines. This contributes for the athlete's performance because the catabolic actions of these hormones supply energy for the active tissues. After the end of a training session or game these hormones must return to basal levels, giving place to the anabolic hormones that will orchestrate the recovery processes (Hackney, 1999; Kellman & Kallus, 1999; Kellmann, 2002). The consequence of the correct balance between the catabolic and anabolic hormones is adaptation with improvement of the physical performance (Budgett, 1998) as well as high feeling of compensation (Fry, 1992; Steinacker & Lehmann, 2002). Therefore, monitoring the stress hormones levels during sports practice may contribute to define the best training program in an individual physiological basis.

In order to address this issue, saliva rather than blood markers are better suitable. The saliva sample collection is painless and the volunteer may easily collect several saliva samples during the day. Moreover, saliva samples may be collected immediately before the athletes get into or off the court, as well as during the game intervals. The salivary hormone concentration is proportional to the concentration of its free fraction in the blood (Vinning & McGinley, 1987) and, in the case of cortisol it is independent of the salivary flow (Kirschbaum & Hellhammer, 1994; Duclos et al., 1998).

The plasma and salivary cortisol levels follows a circadian rhythm that produces a high peak during morning hours progressively declining as the day follows and reaching values next to zero around midnight. This rhythm may be altered by normal daily situations or the presence of stressor agents. Although there are no pre-existing established values of salivary cortisol levels during the awakening hours, the reported values for healthy adults are of about 16.6 nmol/L (Clow et al., 2004). It is also accepted that the early morning hour's peak is a more conservative biological measure than daytime values, although measuring and understanding these values as well the rhythm of cortisol still is an open question in the literature (Wust et al., 2000; Edwards et al., 2001).

Variations of plasmatic or salivary cortisol levels have been used as biological indicators of stress related to several sports. We have reported that football players show cortisol serum concentrations near to the maximum of the normal range (467.4 ± 30.6 nmol.L^{-1}) during the competitive season, and that they increased even more by the end of the season (692.4 ± 41.5 nmol.L^{-1}), decreasing after the playoffs (565.5 ± 46.8 nmol.L^{-1}). These results

indicate that during this period, the team experienced a predominant catabolic state, probably caused by the high levels of cortisol as a consequence of the intensive training and sequence of tournaments with no intervening resting periods.

The competitive basketball is an intermittent high-intensity physical activity that requires a well-development physical. Specific basketball practice is purportedly the best method of improving the fitness characteristics of basketball athletes (Trninic, Markovuc, & Heimer, 2001). Other factors that can influence demands of basketball include game tempo, quality of opposition, style of play, and recovery intervention used by the coach. Over the competition season this combined factors elicit a variable patterns of response (positive, neutral or negative) depending on the adaptability of individual (Montgomery et al., 2008).

The aim of this report is to investigate if the salivary cortisol levels are altered in the days of games during a competitive basketball season as well as whether the salivary cortisol levels immediately before and after the games are related to the athletes' performance during the game.

Methods

Subjects

Ten adult male athletes of a local basketball team volunteered to participate. The study was approved by the Internal Review Board of the School of Medical Sciences, State University of Campinas (UNICAMP), Campinas, São Paulo, Brazil, and it was conducted according to the principles of the Helsinki Declaration. A formal permission to work with the team was received from the team's head coach. Prior to data collection athletes reported the use of no medications, and signed the Informed Consent Term.

The athletes were 19 to 27 year-old (21.20 ± 0.79), 1.78 to 2.00 m height. The team use to train during two hours a day from Monday to Saturday and participates in competitions all over the year. We monitored this team during three months from March to May. During this period, the team participates in a tournament of eighteen games occurring on Wednesdays and on Saturdays or Sundays at 11:00, 16:00, or 20:00 o'clock. During this tournament the team played with two other teams. If the first game took place in the team's court, the next one took place on the opponent's court.

Physical performance testing

Physical performance was tested on a previously determined morning in the club gymnasium at the end of the season. All the players were familiar with the environment and the tests, which were conducted under the supervision of the team coaches and the investigators.

Following instructions, the subjects performed four sprints by the way draft on the court with cones. After the two sprinters without ball, the athletes rest by three minutes when were analyzed lactate concentration. Then, the athletes were repetition this test with ball, by two times following. Again, after three minutes of rest the lactate concentration were realized.

Experimental approach

The athletes were given written and verbal instructions on how to collect saliva samples and received plastic bags containing four salivettes® each (Sarstedt, Numbrecht, Germany). The athletes were requested to collect their saliva samples immediately upon waking up, 30 min after that, and right before going to sleep. When the game was held in the morning (11:00 o'clock) they should collect a saliva sample right before dinner too. When the game was held in the afternoon or at night, they were asked to collect an additional saliva sample right before lunch. Immediately before and after the games, the saliva samples were collected in the court in the presence of the researchers. They were strongly recommended to rigorously follow the instructions and not to brush their teeth or have anything in the mouth but water for at least 30 min before taking any sample. After saliva sampling, the salivettes® were put in the home freezer or refrigerated boxes and transported to the laboratory where they were frozen (-20° C).

Salivary cortisol levels assay

At the time of analysis, the samples were thawed at room temperature and centrifuged (4° C, 20 min, 2800 rpm). The cortisol levels were assayed in duplicate using a commercial immunoassay kit (Diagnostic System Laboratories Inc., Webster, TX, USA). The intra- and inter-assay variations for cortisol were 5 and 8%, respectively.

Statistical Analysis

Data are presented as means ± s.e.m. Student's *t* test, one-way analysis of variance or Kruskal Wallis test were used for comparison between games. When a significant F value was obtained, the Tukey or Dunn tests were used to locate the differences between means. The level of significance was set at $p<0.05$.

Results

The athletes' physical performance was accessed once at the end of the season. The Y test evaluated the athletes' skills without and with the ball. The time spent in the test without ball was 8.85 ± 0.08 s and in the test with ball it was 9.11 ± 0.17 s. The blood lactate measured immediately after each test did not change (6.83 ± 0.44 and 6.85 ± 0.043 nmol/L).
Table 1 presents the games timetable and results. The team won 6 from 18 games.

Table1. Games timetable, place where the games took place, if in the team's house (H) or in the opponent's house (O) and if the studied team has won (W) or lost (L).

Games	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Day	19	22	25	29	31	1	8	16	20	22	26	29	3	10	17	21	24	27
Month	03	03	03	03	03	04	04	04	04	04	04	04	05	05	05	05	05	05
time (h)	11	20	18	20	20	20	18	16	20	11	20	16	20	20	20	16	20	20
H/O	H	O	H	O	H	O	O	H	O	O	H	H	O	O	H	H	O	H
W/L	W	L	W	L	L	L	W	L	W	D	D	D	W	L	L	L	L	W

Table 2 shows the salivary cortisol levels in the days of the games that the athletes did return at least 5 of the six required saliva samples. The saliva samples were required to be collected upon waking up, 30 min after that, and at 23:00 o'clock on all game days. Other two samples were collected before and after the games: at 11:00 and 13:00 o'clock, respectively before and after the games 1 and 10; at 16:00 and 18:00 o'clock for games 8, 12 and 16 that took place at 16:00; and at 20:00 and 22:00 o'clock for the games that took place at 20:00.

Table 2. Mean (± SEM) salivary cortisol levels (nmol/L) of basketball male athletes, at pointed hours, on game days. The saliva samples were collected at hours indicated on table.

Games	8h	11h	12h	13h	16h	18h	20h	22h	23h	n
1	32.86±6.61	32.73±3.49		53.65±4.91			14.63±3.06		10.44±2.74	7
2	31.80±4.54		21.03±3.43				19.59±3.44	34.36±4.39	12.22±2.59	8
4	21.12±4.99		8.68±1.55				9.52±3.89	21.41±6.68	3.96±2.02	8
8	21.86±2.9		20.86±2.89		17.12±2.72	32.07±6.31			10.57±3.97	7
9	16.86±3.66		18.02±2.98				11.02±3.09	19.13±3.85	0.87±0.49	6
10	20.70±5.79	15.03±3.52		21.27±5.47			5.87±2.04		3.91±3.14	6
13	11.85±2.99		9.05±1.82				7.09±1.47	15.17±2.75	4.89±0.65	9
15	12.42±3.13		9.41±2.23				12.86±1.93	26.22±3.59		8
16	16.29±2.57		10.09±2.07		10.02±3.01	19.20±7.04				6

The salivary cortisol levels during the days of the games 1 and 10, both at 11:00 o'clock, are shown in the Figure 1. The game 1 took place at the team's home and they won. The game 10 took place in the opponent's court and the evaluated team lost. On the day of the first game, the salivary cortisol levels were high in the morning upon waking up and 30 min later, remained high until 11:00 o'clock, increasing even more at 13:00. Afterwards they decreased. On the day of the game 10, the salivary cortisol levels showed a similar time course, although in a lower flattened level during all day, with a significant difference only at 13:00 o'clock, immediately after the end of the game (Table 2).

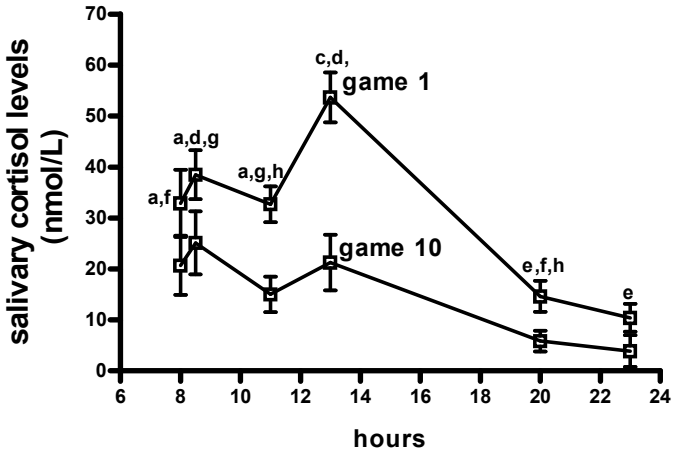

Figure 1. Mean (±SEM) salivary cortisol levels (nmol/L) in basketball male athletes in two days that the game took place at 11:00 o'clock. Different letters show statistical significant differences (ANOVA, plus Tukey test, $p < 0.05$).

In those days that the game occurred at 16:00 (Figure 2) again the cortisol rhythm has been preserved, and the hypothalamus-pituitary-adrenal axis response to the game can be observed as well.

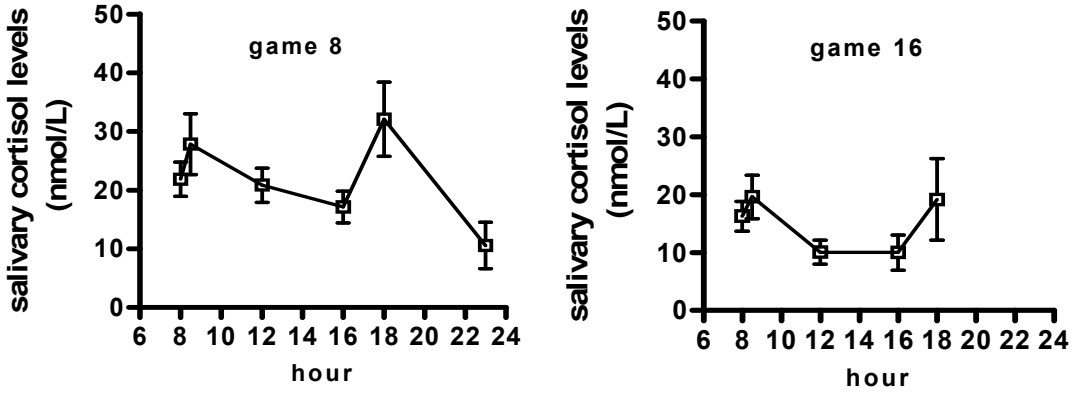

Figure 2. Mean (±SEM) salivary cortisol levels in basketball male athletes during the day of games 8 and 16, played at 16:00 o'clock. * Difference statistically significant between salivary cortisol levels at 23:00 and 8:30; + between 23:00 and 22:00 (ANOVA, plus Tukey test, $p < 0.05$).

The games number 2, 4, 9, 13, and 15 started at 20:00 o'clock. On games 2 and 4 (Figure 3) the cortisol presented normal rhythm in the morning. Right before the game (20:00)

the salivary cortisol levels were similar to those seen at noon, which it is higher for this time and it was even higher at 22:00, decreasing after the end of the game (23:00). On days of the games 9, 13, and 15 the salivary cortisol levels were lower in the morning than they were in the days of games 2 and 4 and didn't decrease along the day, as presumed (Figure 3). Thus, the profile of cortisol rhythm was flat. However, it increased before and during the game suggesting that there was an activation of the hypothalamus-pituitary-adrenal axis by the challenge of the game (Figure 2). Moreover, there is an anticipatory increase in the salivary cortisol levels which remain elevated until after the game has been finished.

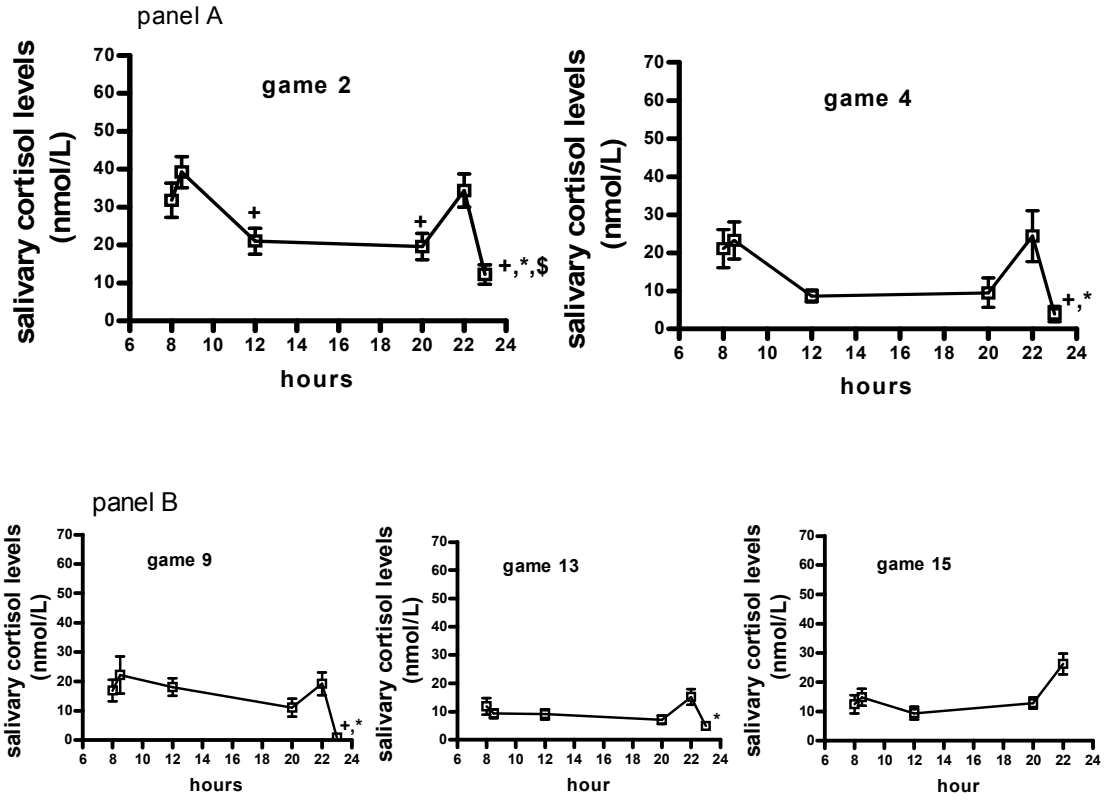

Figure 3. Mean (±SEM) salivary cortisol levels in basketball male athletes during the days of games 2 and 4 that occurred at 16:00 o'clock (panel A), and 9, 13 and 15 that occurred at 20:00 o'clock (panel B). * Difference statistically significant between 12:00, 20:00, 23:00 and 8:30 o'clock; + between 23:00 and 22:00 o'clock; $ between 23:00 and 8:00 o'clock, in panel A (ANOVA, plus Tukey test, $p < 0.05$).* Difference statistically significant between 23:00 and 8:30 o'clock; + between 23:00 and 22:00 o'clock, in panel B (ANOVA, plus Tukey test, $p < 0.05$).

The area under the curve (AUC) of cortisol was higher on game day 1, probably because this was the first game of the tournament and it has been played in the morning, thus resulted in higher increase of salivary cortisol levels than the increases observed on afternoon and night games. The cortisol AUC progressively decreased in subsequent games, except for

the game 8 (Figure 4). There is a positive correlation between salivary cortisol levels before and after the games and it is independent of the game timetable (Figure 5).

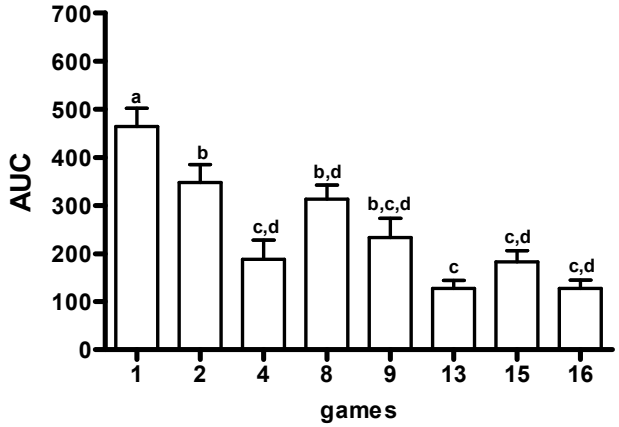

Figure 4. Area under the curve of salivary cortisol (nmol/L) in basketball male athletes on game days. Different letters indicate statistically significant differences (ANOVA, plus Tukey test, $p < 0.05$).

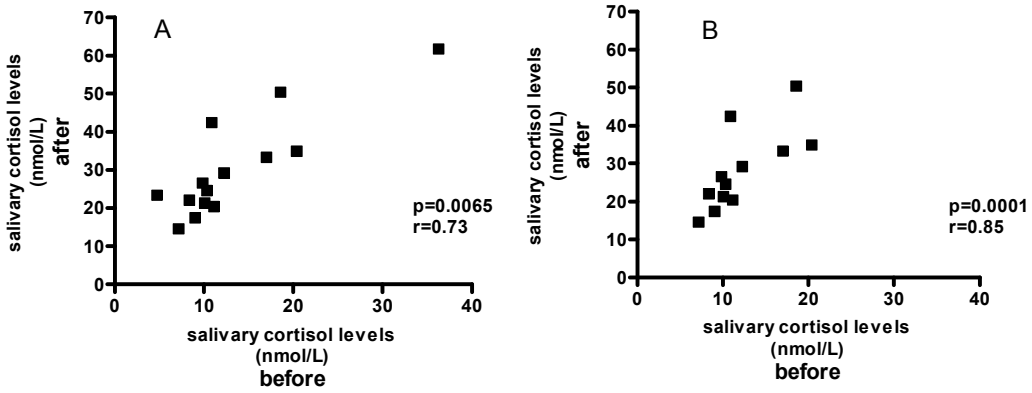

Figure 5. Correlation between salivary cortisol levels (nmol/L) of male athletes before and after basketball games played at 11:00 (panel A) or at 16:00 and 20:00 o'clock (panel B).

Figure 6 shows the salivary cortisol levels response to the games. During the first game (panel A), that took place at 11:00 o'clock on the team's home with victory, there was a significant increase in the salivary cortisol levels. On game 10, played at the opponent's court, which the opponent team won, this difference was not significant. During the games played at 16:00 o'clock (panel B) in the home court, which ended with the studied team defeat, there was no significant increase in the salivary cortisol levels. On panel C are the games played in

the evening. There are no significant increases in the salivary cortisol levels independent of the games results.

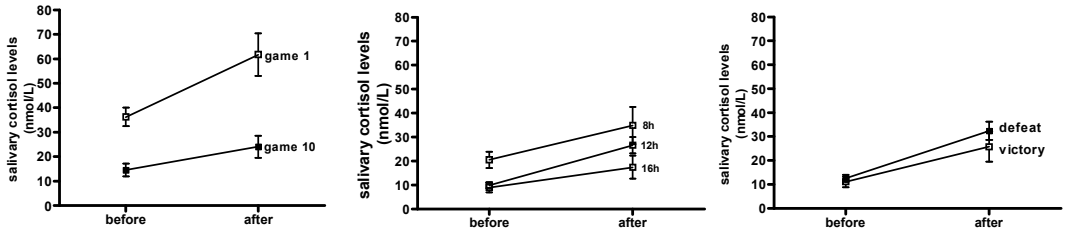

Figure 6. Salivary cortisol levels (nmol/L) in basketball male athletes before and after games played at 11:00 (panel A), 16:00 (panel B) and 20:00 (panel C). Different letters indicate differences statistically significant (ANOVA, plus Tukey test, $p < 0.05$).

The awakening cortisol response (ACR, nmol/L) of the athletes was negative on the day of game 13 and positive on the other games (Figure 7).

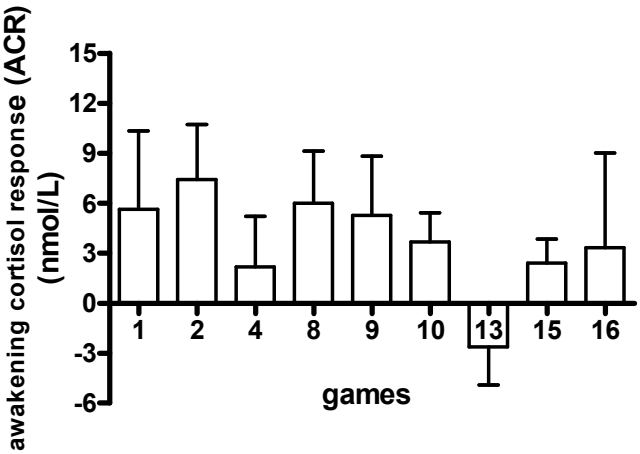

Figure 7. Awakening cortisol response (ACR, nmol/L) of basketball male athletes on game days. There are no statistically significant differences between the days (ANOVA, plus Tukey test, $p > 0.05$).

Discussion

The data presented here have shown that cortisol basal levels were higher in the beginning than along the tournament season, since on the days of the first and the second games cortisol salivary levels were higher compared to the other games. Comparing the cortisol salivary levels in the day of games 1 and 10, both played in the morning, it is possible to see this difference. In the day of the game 10 the cortisol time course was flat, comparing with game 1. Nevertheless, the stress response to the game was still present. The same rational is applicable to games 2 and 4, both played at 20:00 o'clock. On the games that occurred at

16:00, the salivary cortisol levels were lower in the morning and didn't decrease during the day, but the cortisol response before and during the game still remained, although with a flat rhythm.

Usually, the stress response represented by an increase in the cortisol levels is superposed to the basal circadian rhythm (Dugue et al., 2001; Ockenfels et al., 1995), as it can be observed in the results presented here. In all games, independently of the period that they were played or the cortisol rhythm observed, occurred an increase of cortisol levels before the team got into the court, and those levels remained high until the end of the game. Moreover, there was a positive correlation between salivary cortisol levels before and after the games. Moreover, in the morning games the increase in the salivary cortisol levels was higher in the game that the team won than in the game that the team was defeated. This relationship was not seen in games that took place in the afternoon or at night. There was no correlation between the place where the games took place and victory or defeat.

O'Donnel et al. (2008) suggested that may have a relationship between positive affect, with the feeling of psychological well being and the slope of cortisol decline along the day. We haven't analyzed the psychological profile of the athletes, but the constant environment of challenge and dispute experienced by them, mostly the negative affect related to defeat, such as the impossibility of recovery of the team classification in the tournament, as games went on, may be related to the flat profile of the circadian rhythm of cortisol that it was observed.

The area under the curve (AUC) reinforces our hypothesis that the decrease of the salivary cortisol levels during the tournament is related to lower stress levels or negative affect, except for the game 8, where the AUC was higher. In this specific game, the opponent team was one of the best teams taking part in the tournament and the day before was marked by a stressfull event caused by the death of one teammate father.

Based on the discovery that there is a natural increase of cortisol secretion immediately after awakening, and that it hits its peak around 20 to 45 minutes right after, a cortisol awakening response has been described. This corresponds to the difference between the cortisol levels 30 minutes after awakening and the awakening cortisol levels (Clow et al., 2004). It has been considered as being less variable than the cortisol levels in other periods of sleep-wake up cycle (Edwards et al., 2001a, Williams et al., 2005).

In the first 30 minutes after awakening, the salivary cortisol levels increases between 50% and 160%, corresponding to 9 (3.9 – 14.9) nmol/L (Pruessner et al., 1997; Kirschbaum &

Helhammer, 2000; Clow et al., 2004). Some authors classify as “responders” the subjects that present an increase higher or equal to 2.4 nmol/L and suggest that some people do not present this response (Thorn, et al., 2006).

On the other hand, the use of an electronic device to measure the adherence to the temporal sequence of sampling of saliva during the day, demonstrated that the lack of adherence was of 26 – 29 % to participants that have not been informed that they were monitored (Kudielka et al., 2003; Broderick et al., 2004). Not considering the strategy used to control the adherence, the studies have demonstrated that non adherent participants present a reduced CAR.

The athletes presented positive CAR in most of game days, and a negative value on game 13. On game days in which it was positive, the CAR was higher than on resting days (data not show). The higher CAR on week days (represented in our work on game days) in relation to the ones during weekends was related to the higher stress experienced on week days (Kunz-Ebrecht et al. 2004a; Scholtz et al. 2004). We also have detected higher CAR on week days than on weekends on Brazilian executives (Garcia et al, 2006). The magnitude of CAR was positively related to chronic stress (Schulz et al., 1998; Wust et al., 2000a), work stress and depression (Williams et al., 2005). Waking up earlier on work days may be associated to stress at work or changes in the sleeping pattern, causing changes in the circadian rhythm of cortisol (Melamed et al., 1999; Akerstedt et al., 2002). Some authors suggest that people who wake up earlier would have a higher CAR and experience more stress (Edwards et al., 2001b; Kudielka & Kirschbaum, 2003; Federenko et al., 2004). Dettenborn et al. (2007) suggested that sleeping disturbance alters the cortisol levels on the rest of the day, although after an interrupted night of sleep the CAR does not present any changes.

On the other hand, most of the authors suggest that the absence of CAR it has no relationship to awakening time (Steptoe et al, 2004), but in fact it seems related to the lack of compliance to the protocol (Kudielka et al., 2003; Broderick et al., 2004; Kunz-Ebrecht et al. 2004; Wright & Steptoe, 2005; Kupper et al., 2005; Clow et al., 2004; Garcia et al., 2006; Thorn et al., 2006), mainly after waking up. The CAR is very much affected, mainly by the delay on making the first collect (Kunz-Ebrecht et al., 2004; Dockray et al., 2008), artificially causing a drop on the morning peak (Dettenborn et al., 2007).

Nevertheless Pruessner et al. (1997) have admitted that around 10% of the subjects fail to exhibit a normal CAR, independently from the compliance to the protocol of sample

collection. Although many different authors agree on this hypothesis, Lasikiewicz et al. (2008) proposed that the blunt of CAR followed by the flattened of the diurnal rhythm of cortisol corresponds to a physiological profile of cortisol secretion that may be found in some subjects, and not as a result of a methodological error or related to non adherence to the protocol.

O'Donnell et al. (2008) worked with psychological coping styles and the cortisol over the day in healthy older adults, and verified that CAR average was 0.31 μg/dL, equivalent to the increase of 51%, which was similar with other works (Pruessner et al., 1997; Wust et al., 2000a; Clow et al., 2004). The exaggerated CAR was related to a high perceived stress index (Schultz et al., 1998; Wust et al., 2000a). We have found negative correlation between the perceived stress index and the CAR, as compared to groups of subjects with low and high socio-economical status (Garcia et al., 2008), considering that the first group also presented higher indexes of perceived stress and basal levels of cortisol along the day. The low levels of CAR and even a negative CAR value in the day of game 13 suggest that the athletes' compliance to the protocol was poor or that in the athletes te CAR is lower than in sedentary people.

Therefore, the results presented here pointed out those basketball male athletes presented variations in the salivary cortisol levels during the competitive season. The games that took place in the beginning of the season were followed by higher cortisol levels that decreased progressively along the tournament. Independently of the basal level in the day of the game there was an increase of the salivary cortisol level which enhanced even more until the end of the game. It was not possible to establish a correlation between the salivary cortisol level and the game results when the game was played in the afternoon or at night. The intensity of the increase on the salivary cortisol levels was positively correlated to victory in games played the morning.

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TERCEIRO MANUSCRITO:

STRESS LEVELS IN STUDENTS PREPARING FOR UNIVERSITY ENTRANCE EXAMS IN THE STATE OF SÃO PAULO, BRAZIL

STRESS LEVELS IN STUDENTS PREPARING FOR UNIVERSITY ENTRANCE EXAMS IN THE STATE OF SÃO PAULO, BRAZIL

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Short title: Stress levels in Brazilian university students

Abstract

The aim of this work was to evaluate the stress levels in Brazilian students during the year of preparation for entry into public universities. In Brazil, the school year starts in March and finishes in November, with a one-month winter vacation in July. The exam period is in November/December, before the summer vacation. Eighty-one students (26 males and 55 females; mean age, 18.7 ± 0.2 years old) volunteered for the study. Saliva was collected once a month at 8:00 am, 12:00 am and 6:00 pm, from April to November, to determine the salivary cortisol levels as an index of stress. The symptoms of perceived stress (ISS), depression and anxiety were evaluated using appropriate, self-rated inventories in March (M), June (J) and November (N). The salivary cortisol levels were lowest in April (0.62 ± 0.05, 0.40 ± 0.06 and 0.19 ± 0.03 μg/dl, at the three daily samples, respectively) and August (0.75 ± 0.07, 0.30 ± 0.05 and 0.26 ± 0.06μg/dl), and highest in May (1.94 ± 0.23, 1.04 ± 0.14 and 0.87 ± 0.16 μg/dl), June (1.24 ± 0.09, 0.47 ± 0.03 and 0.21 ± 0.03 μg/dl) and September (2.64 ± 0.4, 1.57 ± 0.34 and 0.20 ± 0.02 μg/dl), the months when students register for the universities they intend to enter. The salivary cortisol levels were higher in November (0.88 ± 0.10, 0.30 ± 0.05 and 0.16 ± 0.04 μg/dl) than in April and August, but lower than in May, June and September. Most of the students showed changes in their cortisol rhythm during the year. There was a significant Spearman negative correlation between salivary cortisol levels and exam performance, with the higher cortisol levels related to no approval in the exam (Spearman r = -0.28; IC = -0.48 to -0.06; $p < 0.01$). The mean scores (± standard deviation) were 34.1 ± 9.3 (M), 34.9 ± 9.3 (J) and 35.6 ± 10.1 (N) in the Inventory of Stress Symptoms (ISS), 50.5 ± 8.3 (M), 51.7 ± 9.2 (J) and 54.2 ± 10.5 (N) in the self-rated anxiety scale, and 53.5 ± 10.1 (M), 53.2 ± 9.4 (J) and 56.2 ± 10.6 (N) in the self-rated depression scale. We conclude that stress, depression and anxiety are pronounced in Brazilian students preparing for university entrance exams. The salivary cortisol levels oscillate during the year, with higher values in the months when the students are choosing their courses and university. These symptoms are enhanced at the end of the academic year, immediately before the exam period.

Key words: examination period, performance, salivary cortisol, stress, students

Introduction

Exam periods are times of great stress for students. In Brazil, university entrance exams are particularly important for many students because studying at a public university will guarantee the best job opportunities in the future. However, it is very hard to pass these exams, particularly for courses such as the medical sciences, biology, electrical engineering and law, in which the candidate:vacancy ratio can be as high as 80:1. Therefore, students usually prepare for these exams during an additional year after finishing high school. This is a year of considerable intellectual work and psychological stress, including several hours of daily study, endless classes, and the pressure to meet the teachers´ and family's expectations. However, so far, no studies have examined the levels of stress during this period of student life.

There is now considerable evidence that chronic stress can result in allostatic overload (McEwen and Wingfield, 2003), and that stress hormones such as corticosteroids and adrenaline can impair memory and cognition in humans and animals (Lupien and McEwen, 1997) and influence performance. Nevertheless, there are only limited and conflicting data on the cortisol levels in students during the exam periods and the influence of these levels on performance. Vedhara et al. (2000) showed that during the exam period undergraduate students had high levels of perceived stress that were associated with low levels of salivary cortisol and improved performance in tasks involving short-term memory, but poor performance in tasks measuring selective and divided attention. Fontani et al. (2004) reported that individuals with high cortisol levels had an increased reaction time to stimuli that required decision making.

The aim of this study was to quantify the salivary cortisol levels in students during the year they were preparing for university entrance exams in order to determine whether there was a correlation between the levels of this hormone and performance in the examination. We also used an inventory of stress symptoms and self-rating scales for depression and anxiety to evaluate the presence of stress symptoms and their correlation with salivary cortisol levels and academic performance.

Methods

Experimental subjects

Volunteers (26 male and 55 female; 16-24 years old) were recruited from among the students of a course preparatory for exams located in Araraquara, a city of 400,000 inhabitants about 300 km northwest of São Paulo, the state capital. The classes of this course start in March and continue until November, with exams in June and November.

The volunteers and one of their parents signed a term of informed consent, as well as a health declaration. Students who declared any pathology or who were using any medication were excluded. The volunteers were free to abandon the study at any time, without any restriction or penalty. Students who left the course during the year were automatically excluded from the study. The results obtained were not usually made public, but students could request a report of their data and the mean values for comparison.

The experimental protocol was approved by the institutional (UNICAMP) Ethics Committee for Human Research (nº 604/2002) and was done in accordance with the recommendations of the Declaration of Helsinki.

Salivary cortisol assay

Saliva was collected in Salivettes® (Sarstedt, Numbrecht, Germany). The students were shown how to introduce a piece of cotton into their mouth and keep it there until saturated with saliva (about 2 min). The piece of cotton was subsequently returned to a plastic tube that was stored in the refrigerator until the next day when the samples were taken to the lab, centrifuged and frozen (-20^{o}C) until the salivary cortisol levels were determined by enzyme immunoassay (Diagnostic Systems Laboratories Inc., Webster, TX, USA).

The participants were told to collect three samples of saliva at 8:00 am (soon after waking up and washing their mouth and before breakfast), at 12:00 am (before lunch) and at 8:00 pm (at least 2 h after dinner). The samples were collected once a month, starting in the first week of April until the first week of November. In July, no samples were collected since some students were doing exams and others were on vacation. A strong recommendation was made to avoid contamination of the saliva samples with blood.

Psychological evaluation

The questionnaires for perceived stress, Inventory of Stress Symptoms (ISS), (Lipp, 1987), depression (Zung, 1965) and anxiety (Zung, 1971) were answered at home in March, June and November. The ISS was validated for the Brazilian population and consisted of 14 items in which the respondent could choose one of the following options: (1) never, (2) sometimes, (3) regularly, (4) frequently, and (5) very frequently. The response scores were summed and varied from 14 (no stress at all) to 70 (massive presence of stress symptoms) (Rocha-Pinto, 1996).

The Zung scale for symptoms of depression included a list of 20 items. Two items referred to persistent affective symptoms, such as depression, sadness, melancholia and crying. Eight items referred to the physiological equivalents of these affective symptoms (diurnal variation with exacerbation of the symptoms in the morning and alleviation during the day, frequent and early waking up, anorexia, body weight loss, lowering of libido, bowel constipation, tachycardia and fatigue). Ten items were related to the psychological equivalents of these symptoms, such as psychomotor agitation, psychomotor retardation, a sensation of emptiness, helplessness, indecision, irritability, self-deprecation and thoughts of suicide). The respondent marked one of four alternatives for each of the foregoing items as follows: (1) rarely or never, (2) sometimes, (3) frequently and (4) always. The final index was obtained by the sum of the values and converted to an arbitrary scale of 100. A low index indicated no depression, whereas high scores indicated significant clinical depression.

The self-rated anxiety scale (Zung, 1971) is based on similar premises and methodology to the Zung scale. The anxiety scale offers 20 items related to affective and somatic symptoms in which individuals with less anxiety have lower scores and those with an important degree of anxiety have higher scores.

Statistical analysis

The salivary cortisol levels (μg/dl) were expressed as the mean ± standard error of the mean (sem). The area under the curve (AUC) was determined for the awaking day period by using the three samples obtained and then applying the trapezoid rule, with the base of the trapezoid being zero (Pruessner et al., 2003). The scores in the inventory of stress symptoms

and the self-rated scales for depression and anxiety were expressed as the mean ± mean standard deviation (SD).

Data with a normal distribution were compared using Student's t-test for paired samples, or analysis of variance (ANOVA) for repeated measures, followed by the Tukey test. Data with a non-Gaussian distribution were compared using the Friedman text followed by the Dunn test. Pearson correlation tests were applied to data with a Gaussian distribution and the Spearman correlation was used for data that did not have a normal distribution. Values of $P<0.05$ were statistically significant. All statistical analyses were done using Prism GraphPad software.

Results

The volunteers (119 out of 220 students enrolled in the preparatory course) were recruited through a seminar in which the aims of the project were explained. During the year, some of the students left the course whereas others did not meet all of the criteria for inclusion. As a result, only 81 students were included in the study.

Table 1 shows the salivary cortisol levels and the area under the curve for salivary cortisol in each month during the academic year that preceded the university entrance exam. Since there were no differences related to the gender or career (data not shown), these parameters were not considered for further analysis. The cortisol levels of salivary samples collected at 8:00 a.m. in April and August, the beginning of the academic semesters, were 0.62 ± 0.05 and 0.75 ± 0.07 μg/dl, respectively ($P>0.05$; ANOVA for repeated measures plus Tukey test). However, in May, June and September, the salivary cortisol levels were significantly higher than in the other months ($P<0.05$; ANOVA for repeated measures plus Tukey test). In October and November, the values decreased but were still higher than in April and August. There were no differences in the salivary cortisol levels among the samples collected at 12:00 a.m. and 8:00 p.m. in April, June, August and November ($P>0.05$, ANOVA for repeated measures plus Tukey test). However, these levels were significantly lower than those in samples collected in May (both samples) and September (samples collected at 12:00 am).

Table 1. Salivary cortisol levels (μg/dl) and area under the curve (AUC) for salivary cortisol during the day (μg/dL) in students preparing for university entrance exams.

Month	8:00 am[#]	12:00 am[#]	8:00 pm[#]	AUC*
April	0.62 ± 0.05[a]	0.40 ± 0.06[e]	0.19 ± 0.03[i]	4.01 ± 0.44[h]
May	1.94 ± 0.23[b]	1.04 ± 0.14[f]	0.87 ± 0.16[j]	7.62 ± 0.71[k]
June	1.24 ± 0.09 [b]	0.47 ± 0.03[e]	0.21 ± 0.03[i]	5.30 ± 0.37[h]
August	0.75 ± 0.07[a]	0.30 ± 0.05[e]	0.26 ± 0.06[i]	3.72 ± 0.42[h]
September	2.64 ± 0.40[c]	1.57 ± 0.34[f]	0.20 ± 0.02[i]	7.13 ± 0.84[k]
October	0.80 ± 0.11[d]	0.53 ± 0.05[g]	0.48 ± 0.17[j]	5.32 ± 0.56[h]
November	0.88 ± 0.10[d]	0.30 ± 0.05[e]	0.16 ± 0.04[i]	4.15 ± 0.41[h]

Values are the mean ± sem of 81 samples; [#]Time of saliva collection; *Area under the curve calculated by the trapezoidal method. Values followed by different letters are significantly different (P<0.05, ANOVA followed by the Tukey test).

Figure 1 shows the area under the curve for the salivary cortisol levels in each month of the academic year.

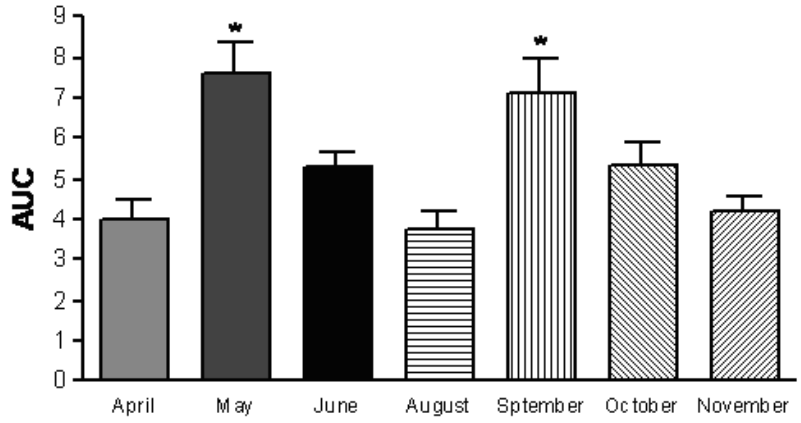

Figure 1. Area under the curve for the salivary cortisol levels (μg/dl/h) during the day in students preparing for university entrance exams. Vertical bars represent the means and the sem. *P<0.05 compared to the other months (ANOVA plus Tukey test).

We established ranges of values for the salivary cortisol levels based on the mean salivary cortisol concentration of samples obtained at 8:00 a.m. in April, as follows: values lower than the mean minus the standard deviation (SD; 0.62 - 0.37 = 0.25 μg/dl), values

between the mean minus the SD (0.25 μg/dl) and the mean plus the SD (0.99 μg/dl), values between the mean plus the SD (>0.99 μg/dl) and the mean plus two SD (1.38 μg/dl), values greater than the mean plus two SD. The values in the higher range were considered indicative of cortisol hypersecretion (Lupien et al., 1996). Figures 2 and 3 show the number of students with salivary cortisol levels in each of these ranges at 8:00 a.m. in the first and second semesters, respectively. In May and June, there was a decrease in the number of students with salivary cortisol levels in the lower ranges compared to April (24 and 30 *vs*. 67, respectively), and an increase in the number of students in the range of 1.00- 1.38 μg/dl (13 and 27, respectively *vs*. 6), as well as in the range >1.38 μg/dl (43 and 24, respectively, *vs*. 7) (Figure 2). In August of the second semester, 60 students had salivary cortisol levels in the lower range, 10 had levels between 1.00 and 1.38 μg/dl and 9 had levels >1.38 μg/dl. In September, the values were particularly high, with 51 students in the higher range of cortisol levels. In October and November, there was a decrease in the number of students in the higher ranges of cortisol (24) and an increase in the number in the lower ranges (24 students in both months) (Figure 3).

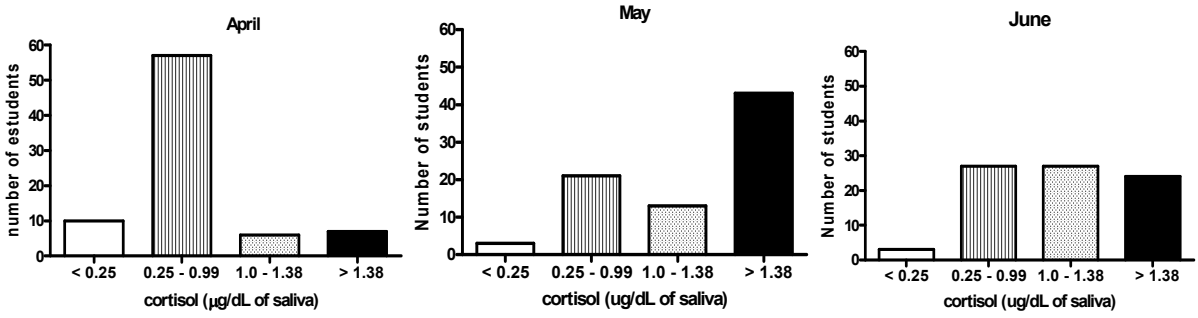

Figure 2. Number of students in each range of salivary cortisol level (μg/dl) arbitrarily defined based on saliva samples collected once a month at 8:00 a.m. during the first academic semester of the year preceding the university entrance exams.

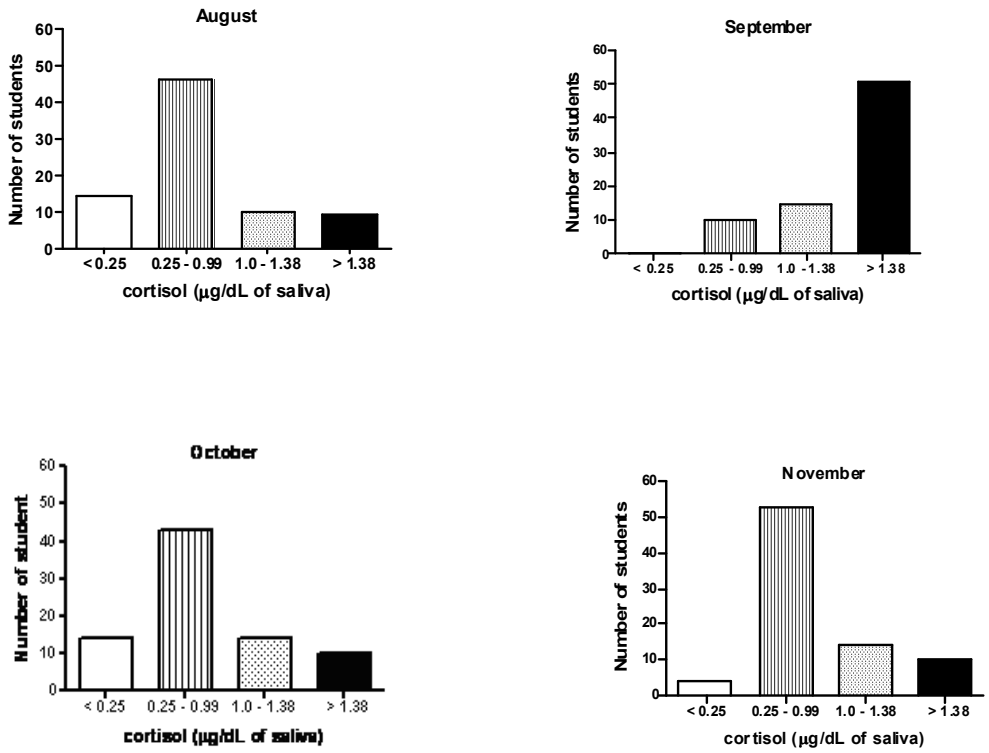

Figure 3. Number of students in each range of salivary cortisol level (µg/dl) arbitrarily defined based on saliva samples collected once a month at 8:00 a.m. during the second academic semester of the year preceding the university entrance exams.

Figure 4 shows the time course of the salivary cortisol levels during the day. As mentioned above, the salivary cortisol levels were higher in May and September, although the circadian rhythm was apparently preserved. The circadian rhythm was considered to be normal when the cortisol concentrations in the samples obtained at 12:00 a.m. and 8:00 p.m. were lower than 75% of the value at 8:00 a.m. (Krieger et al., 1971). Only six students (7%) showed a normal cortisol rhythm in all intervals, whereas the remaining 93% showed some alteration in the rhythm in at least one month. Most of the students showed rhythm alterations in three of the seven months (Table 2); these changes were more frequent at the beginning of the year (April and May) and decreased as the year advanced (Table 3).

Table 2. Number of months in which the cortisol rhythm was normal or altered in students preparing for university entrance exams.

Rhythm	Number of students	%
Normal	6	7
Altered	75	93
1 month	7	9
2 months	16	20
3 months	27	33
4 months	19	23
5 months	6	7

Table 3. Number of students with an altered rhythm of cortisol in each month of the year.

First semester	Students	Second semester	Students
April	54	August	32
May	54	September	20
June	23	October	22
		November	28

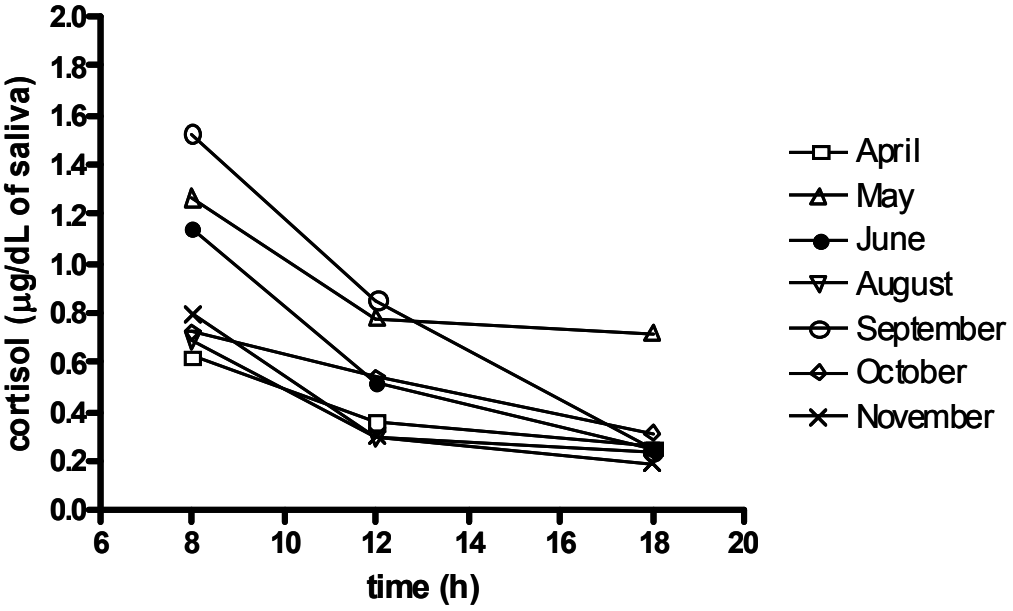

Figure 4. Salivary cortisol levels in students during the academic year preceding the university entrance exams. Samples were collected at 8:00 a.m., 12:00 a.m. and 8:00 p.m., once a month, from April to November. The data are the means of 81 students. The standard errors of the means are indicated in Table 1.

The level of perceived stress and the rates of depression and anxiety were evaluated in March, June and November (Table 4). The ISS consists of 14 items, of which seven refers to cognitive aspects and the other seven to somatic aspects (Lipp, 1987). Each item is scored on a scale of 1 to 5 points so that the minimum score would be 14 for no stress symptoms and the maximum would be 70 for the massive presence of stress symptoms. Individuals are considered stressed when they scored more than 28 (Rocha-Pinto, 1996). The mean score in the ISS was higher than 28 in the three months that the inventory was answered, with no significant differences between the beginning and end of the academic year. Thirty-five students had ISS scores higher than the mean score of the group in March, 41 in June and 36 in November. Forty-five students scored higher in November than in March.

Table 4. Mean scores (± standard deviation) for the Inventory of Stress Symptoms (ISS, Lipp, 1987), self-rating depression scale (Zung, 1965) and self-rating anxiety scale (Zung, 1971) obtained by students in March, June and November during the year of preparation for university entrance exams.

		June	
	March		***November***
ISS	34.1 ± 9.3[e]	34.9 ± 9.3 [e]	35.6 ± 10.1 [e]
Depression	53.5± 10.1[c]	53.2 ± 9.4[c]	56.2 ± 10.6[d]
Anxiety	50.5 ± 8.3[a]	51.7 ± 9.2[a]	54.2 ± 10.5[b]

Different letters indicate significant differences (P<0.05, ANOVA followed by the Friedman test).

Figure 5 shows the number of students according to the range of stress intensity based on the ISS scores. In March, most of the students scored below 28 or between 28 and 42, which indicated low or moderate stress levels, respectively. No student scored more than 56 (high level of stress). However, in June and November, one and three students, respectively, scored higher than 56. The scores in the self-rated scales of depression and anxiety (Zung, 1965, 1971) reinforced these data since both scores were higher in November than in March (Table 4).

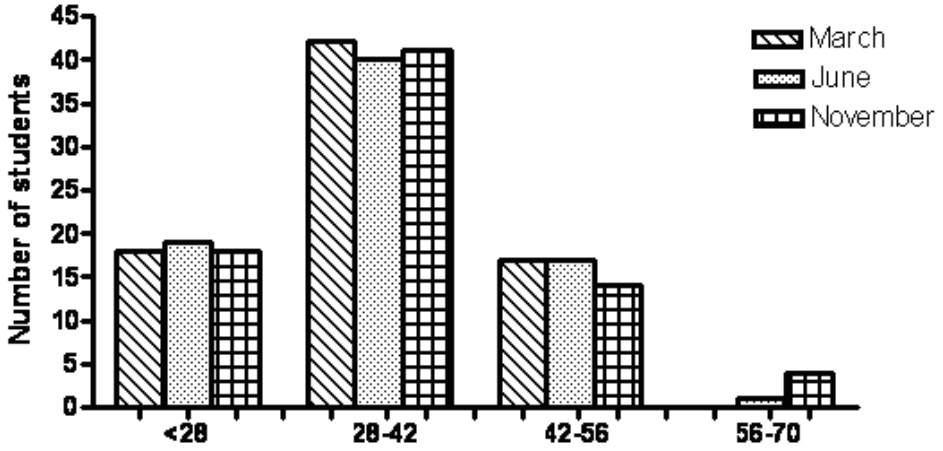

Figure 5. Number of students in each score range in the Inventory of Stress Symptoms (ISS) in March, June and November of the year preceding the university entranc exams.

Eight-one students participated in this project. Of these, 38 passed their university entrance exams while 43 did not. Those who failed returned to the preparatory course the next year or entered in a private university. There was no Spearman correlation between the performance in the exam and the total area under the curve for salivary cortisol or the area under the curve for the salivary cortisol levels in November, the month in which the exams were taken. There was a significant correlation between the salivary cortisol levels in the samples collected at 8:00 a.m. in November and success in the exams (Table 5).

Table 5. Spearman correlation for the exam result (approval *vs*. failing) and the salivary cortisol levels expressed as the area under the curve for the mean value of the entire year (AUC all year), for the salivary cortisol levels in November (AUC November) and for the salivary cortisol level at 8:00 am in November ([Cortisol 8:00 am] November).

	AUC all year	AUC November	[Cortisol 8:00 am] November
r Spearman	0.060	- 0.029	- 0.284
95% confidence interval	-0.17 to 0.28	-0.25 to 0.20	- 0.48 to - 0.064
P value	0.59	0.79	0.01
Significance	no	No	yes

For the test, approval was scored as 1 and failing as 2.

Discussion

Salivary cortisol levels have been accepted as an indicator of plasma cortisol levels and are apparently correlated with the level of stress (Kirschbaum et al., 1995; Vedhara et al., 1999; Bauer et al., 2000). In this work, the salivary cortisol levels were used to indicate the level of stress in students preparing for public university entrance exams in the State of São Paulo (Brazil). These exams occur in July (in a few universities) and November (in all public universities).

As shown here, during the year of preparation for the entrance exams, the highest levels of salivary cortisol were detected in May, June and September. This result indicates that the period of choosing the desired courses and university and the subsequent registration (May

and September) were associated with higher levels of stress than in the period of the exams (November). Nevertheless, the salivary cortisol levels were also higher in November than in April or August, the beginning of the first and second semesters, respectively. A similar finding was seen in June compared to April.

The ISS revealed that in all three months the students scored higher than 28, which meant that they all had symptoms of low to moderate stress (Lipp, 1987). But, there weren't statistically significant differences in the ISS scores in March, June and November. However, in March no students showed symptoms of intense stress or burnout, but this was detected in one student in June and in three students in November.

There was a greater frequency of symptoms of depression and anxiety in November compared to June and March. The scales used here (Zung, 1965, 1971) allowed the identification of symptoms that are frequent in most emotional disorders of clinical relevance and were easy and quick to apply. The correlation between stress and the states of mood or disorders is more pronounced when there is no alternative, with the stress frequently involving frustration (Sapolsky, 2001). When the stress is chronic or long-lasting, as in the case of the students evaluated here, there is a need for constant monitoring so that the person is always alert, thereby triggering anxiety. Alternatively, the stress may not be overcome and can lead to helplessness, even in situations that are manageable, and this may trigger depression (Sapolsky, 2001). In contrast to anxiety, depression is characterized by helplessness, psychomotor slowing and anedonia. Stress causes depression through pathways associated with mood and pleasure, and may reduce the individual´s capacity for attention and vigilance; there may also be alterations to the rhythm and quality of sleep as well as mood.

The students were exposed to high levels of corticosteroids in May, June and September and to moderately high levels of cortisol in October and November. Corticosteroids exert an inverted-U shape influence on the processes of arousal and selective attention, with similar modulatory effects on processes associated with memory, particularly those related to the acquisition and consolidation of the memory trace (Lupien and McEwen, 1997).

Long-term exposure to stress or glucocorticoids impairs memory function in humans (for a review, see Roozendaal, 2002). These impairments are restricted to memory retrieval for spatial/contextual or declarative information. Declarative memory refers to the conscious or voluntary recollection of previously learned information (Milner et al., 1998). Hence, a glucocorticoid rush can impair consolidation of novel information, while simultaneously

impairing the retrieval of stored information. The high salivary cortisol levels seen in May, June and September may have impaired the students´ memory consolidation and their ability to learn new issues. In "normal" cognition, memory consolidation and retrieval processes occur continuously and simultaneously, but independently (Lupien and McEwen, 1997; De Kloet and Joëls, 1999; Roozendaal, 2000).

Although some authors have reported dose-dependent, glucocorticoid-induced memory enhancement, the potentially disruptive effects of glucocorticoids on memory have received much attention (reviewed by Roosendaal, 2002). Memory consolidation is enhanced by post-training activation of glucocorticoid-sensitive pathways that are also activated by catecholamines and requires stimulation of the amygdala (McCaugh, 1996; McCaugh et al., 2000). However, once memories are consolidated, the efficacy or accuracy of the information retrieved remains vulnerable to glucocorticoids at the time of recall. The oral administration of stress-inducing doses of cortisone (a precursor of the endogenous human glucocorticoid cortisol) 1 h before retention testing impaired hippocampal-dependent free recall of previously learned words (De Quervain et al., 2000). Hence, memory retrieval may be temporarily disrupted during a stressful experience such as an academic examination, regardless of whether the corticosteroids levels are excessively high or not.

The two stress indexes used here, namely, the salivary cortisol level and the ISS score, indicated a high incidence of stress in the students during certain months of the preparatory year preceding the university entrance exams, although the salivary cortisol levels were only moderately elevated in the month that the students sat their exams. Although the students did not undergo memory tests, their performance in the exams may be used as an index of their learning and ability to recall learned issues. Cognition is affected by the level of corticosteroids in a selective and inverted U-shaped (Lupien and McEwen, 1997; Lupien et al., 1999). The reduction in cortisol levels observed in November compared to September may not have favours fast decision-making, as well as memory function and attention. Indeed, there was a significant negative correlation between salivary cortisol levels and passing the exam, although the experimental approach used here made it difficult to establish a cause-effect relationship between these parameters. Fontani et al. (2004) reported that individuals with high cortisol levels showed an increased response time to stimuli that required decision making.

As shown here, there was also a high incidence of alterations in the diurnal cortisol rhythm. Although disturbance of the cortisol rhythm has been associated with clinical

depression and post-traumatic stress disorder (Yehuda et al., 1996), the pattern of findings with respect to psychosocial influences such as employment status or workload in healthy individuals is at best mixed (Ockenfelds et al., 1995; Adam and Gunnar, 2001; Grossi et al., 2001). Hence, the alterations in the diurnal cortisol rhythm seen here were probably related to the students' workload throughout the academic year.

We conclude that stress, depression and anxiety are pronounced in Brazilian students preparing for university entrance exams. The salivary cortisol levels oscillate during the year, with higher values in the months when the students are choosing their courses and university. These symptoms are enhanced at the end of the academic year, immediately before the exam period.

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QUARTO MANUSCRITO

SALIVARY CORTISOL LEVELS IN BRAZILIAN CITIZENS OF DISTINCT SOCIOECONOMIC AND CULTURAL LEVELS

SALIVARY CORTISOL LEVELS IN BRAZILIAN CITIZENS OF DISTINCT SOCIOECONOMIC AND CULTURAL LEVELS

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Running title – Salivary cortisol levels and socioeconomic status

Abstract

We have analyzed the perceived stress index (PSI), the basal salivary cortisol levels, and the awakening cortisol response in 85 volunteers of low (LSES) and high socioeconomic status (HSES). The LSES presented higher PSI and basal salivary cortisol levels, non-altered awakening cortisol response or cortisol diurnal rhythm. We have concluded that the LSES is associated with high PSI and salivary cortisol levels (SCL), which could impact negatively in health and it is related to the daily life stress experienced by LSES group. Because about 30% of the total Brazilian population is included in the LSES group it might have a great impact on public health policies and costs.

Key words: stress, socioeconomic status, salivary cortisol levels

Introduction

The socioeconomic status (SES) is considered a well-established risk factor for poor health, the poorer the SES the worse prospects for health outcomes (Taylor et al., 1997; Marmot et al., 1997). SES gradients in disease risk are partially explained by bad health related with behaviors, and partially by environmental conditions that people at lower SES are exposed[3]. There are only a few reports on the relationship between low SES and stress markers in people leaving in developing countries (Cannino et al., 1987; Almeida-Filho et al., 1997; Araya et al., 2003), including the Brazilian population, where the social differences are accentuated, the social support is deficient, and the public violence index is alarmingly high (Andrade et al., 2002).

Stressors whether they were physical or psychological, activate several neural pathways including the hypothamo-pituitary-adrenal (HPA) axis. One of the consequences is the increase in the cortisol plasma levels that superimpose to the endogenous corticosteroids circadian rhythm. The cortisol rhythm might be altered by the low SES (Steptoe et al., 2003)

psychosocial work or domestic environment (Adam et al., 2001), conditions that determine chronic psychosocial stress, and contribute to the prevalence of severe illnesses.

Because there is a good correlation between stress and cortisol plasma levels and between this last with the salivary cortisol levels the analysis of the salivary cortisol levels has been considered a good physiological indicator of the stress response concerning to the activation of the HPA axis (Clow et al., 2004).

In this paper we have analyzed the perceived stress index, the basal salivary cortisol levels and rhythm, and the awakening cortisol response in two groups of different socioeconomic status living in the state of Sao Paulo, southeast region of Brazil, in order to determine whether these levels correlate with SES.

Method

Thirty-five men and 51 women, 20-45 year-old, volunteered to participate in response to a public notification and signed the Informed Consent Term. They were distributed in two groups: low and high socioeconomic status (LSES and HSES, respectively), according to years of formal education, average wage income, occupational level, family profile, and possessions of some items at home (Instituto Brasileiro de Geografia e Estatística, IBGE). All participants reported use of no medication. This study was approved by the Institutional Ethics Committee and it was conducted according to the principles of the Helsinki Declaration.

To assess perceived stress index, the Perceived Stress Questionnaire (Levenstein et al., 1993) was used. The questionnaires were fulfilled by the volunteers in the presence of the researcher.

The participants were given written and verbal instructions on how to collect the saliva samples and a plastic bag containing five collecting tubes (Sarstedt, Numbrecht, Germany).

They were strongly recommended to fast, to refrain from smoking and not to brush their teeth for at least 30 min before taking the samples. After saliva sampling, the salivettes were centrifuged (4°C, 20 min, 2800 rpm) and frozen (-20^{o}C). Saliva samples were collected in the same regular working day immediately after awakening, 30 min after that, at 7:00, 12:00 and 20:00 h.

In the analysis moment, the samples were thawed and cortisol levels were assayed by EIA kits (Diagnostic Systems Laboratories Inc., Webster, TX, USA). The inter-assay and intra-assay coefficient of variation were 6.9 and 6.2%, respectively.

Data are presented as means ± sem. The groups were compared by Student's *t* test or Analysis of Variance and Man-Whitney test and Spearman correlation. Differences were considered significant when $p<0.05$.

Results

Table 1 shows that there was no difference in the mean age between groups, but they are statistically different concerning to monthly income wage and years of education, characteristics of the huge social differences observed in developing countries.

Table 1 –Age and socioeconomic characteristics of the low socioeconomic status (LSES) and high socioeconomic status (HSES) groups

	n	age(years)	monthly income (US $)	monthly income[1]	Formal Education (years)
LSES	42	34 ± 2.5	228.00±15.50	up to 4	5.57 ± 0.54
HSES	44	39 ± 2.2	3650.00±399.00	higher than 5	20.17 ± 0.40

[1] montly income in number of Brazilian minimum wage income = US$ 190.00

The LSES group perceived stress index (0.44 ± 0.04) was higher than the HSES (0.29 ± 0.04) (Fig. 1).

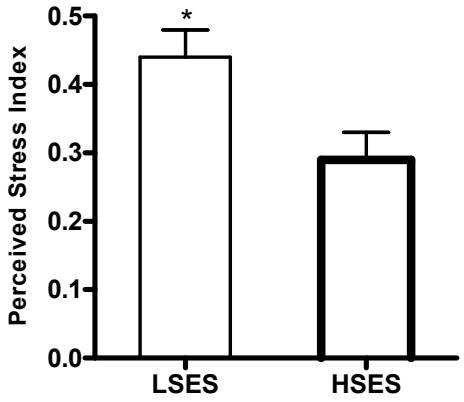

Figure 1. Perceived stress index in the low (LSES) and high socioeconomic status (HSES) groups. * means difference statistically significant between groups (Man-Whitney test, $P<0.05$)

The salivary cortisol levels were higher in the morning than at noon and both were higher than in evening, in both groups, indicating that the cortisol diurnal rhythm has been preserved. The salivary cortisol levels were higher in the LSES group than the HSES group in all the three collected samples (Fig. 2).

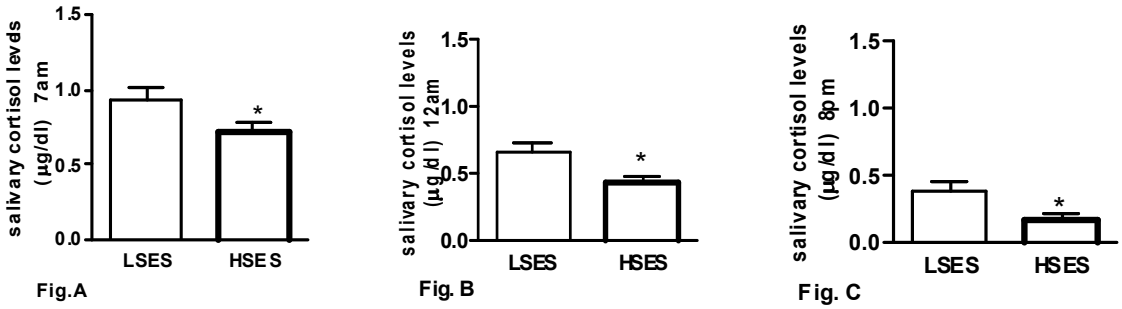

Figure 2. Mean (± epm) salivary cortisol levels (µg/dl) at 7:00 (A), 12:00 (B) and 20:00 (C) h in subjects of the low (LSES) and high socioeconomic status (HSES) groups. * means difference statistically significant between groups (Student's *t* test, $P<0.05$).

Nevertheless, the awakening cortisol response (ACR) (Clow et al., 2004) was not different between groups (Table 2). There was a negative correlation between monthly income wage and salivary cortisol levels, expressed as the area under the curve (Fig. 3).

Table 2. Awakening cortisol response (ACR) in the low socioeconomic status (LSES) and high socioeconomic status (HSES) groups

	N	awakening cortisol	30 min after	ACR
LSES	34	0.86±0.09	1.29±0.10	0.38±0.05
HSES	24	0.68±0.07	1.16±0.08	0.48±0.07

Data are means ± sem of cortisol levels in µg of cortisol/ dl of saliva; n = number of volunteers; * difference statistically significant (P<0.05: Student t test).

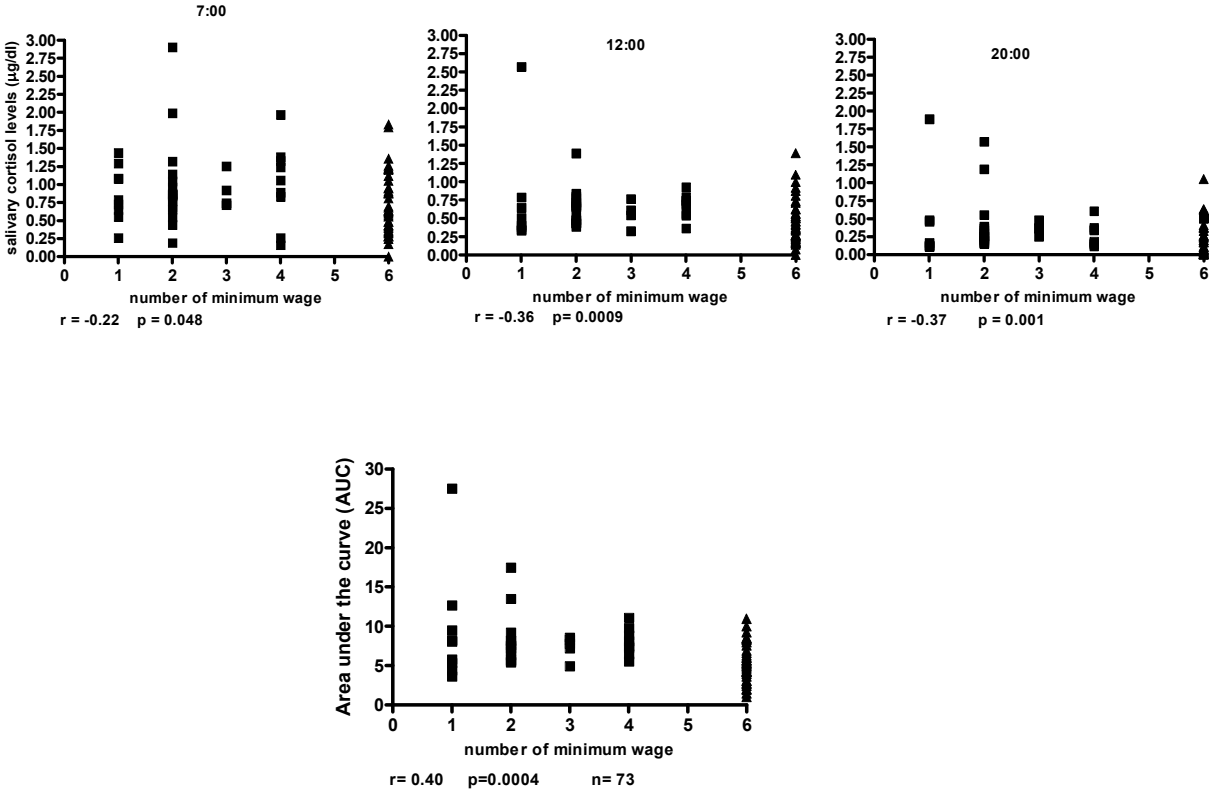

Figure 4. Spearman correlation between number of minimum wage and area under the curve of salivary cortisol (µg/dl) in the LSES (□) and HSES (Δ) groups

Discussion

The results presented here have shown that the LSES group compared to the HSES group presented higher perceived stress index, higher basal salivary cortisol levels with no alteration on the ACR or the cortisol diurnal rhythm. These two populations present a huge difference in the SES, and correspond to the two extremes of the social pyramid in Brazil.

The perceived stress index was higher in the LSES group than in the HSES and higher than the index reported for a healthy Spanish sample (Sanz-Carrillo et al., 2002), but it was similar to that determined in a group presenting physical morbidity (Levenstein et al., 2000).

The social conditions in which these LSES group lives is associated with several daily stressors such as high levels of psychosocial and financial stress, high levels of violence and lower levels of social support. Moreover, stress levels can be partially explained by bad health related to behaviors such as smoking, food choice and alcohol consumption. They present higher basal salivary cortisol levels compared to the HSES group. The differences between these two groups were significant even though considering that the basal SCL present great variability among individuals and from day to day in the same individual.

Different from the basal SCL, the ACR shows a high degree of intra-individual stability (Pruessner et al., 1997) and it has been proposed to measure the level of psychological stress in daily life. There were no differences in the ACR between the LSES and the HSES groups analyzed here. However, it is important to mention that the adhesion to the collecting protocol for ACR is very poor and this index has been questioned in the literature (Levenstein et al., 1993). The salivary cortisol levels were higher in the LSES group than in the HSES in all the three samples collected. The rise in the cortisol in the morning may reflect anticipatory stress, which has been found to be important determinant of cortisol excretion in relation to a stress test (Gaab et al., 2005). The maintenance of high cortisol levels during the day, although declining according to the cortisol rhythm, might be related to the strains that the individual encounters during the day (Smyth et al., 1998). These combined factors create the allostatic load that might generate the conditions to morbidity.

Therefore, we have concluded that the LSES is associated with high levels of PSI and SCL. Considering that the HSES group also includes working men and women living in the same region and country, and exposed to similar general conditions, we might attribute these differences to the SES. Because Brazilian LSES population corresponds 30% of the total population (IBGE) this picture might have a great impact on public health polices and costs. Such impact remains to be analyzed.

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CONCLUSÃO

O cortisol mostrou-se um marcador de estresse, independente do tipo de amostra em que foi quantificado, a invasiva plasmática, ou a não invasiva salivar. Entretanto, amostras salivares apresentam vantagens por permitirem a determinação do ritmo circadiano e da área sob a curva do hormônio. Estas informações representam o diferencial na interpretação dos dados, pois, embora a concentração plasmática de cortisol apresentada pelos jogadores juniores de futebol tenha sugerido um constante estado catabólico, o caráter pontual do dado, limita a sua interpretação. Por outro lado, os dados obtidos em atletas masculinos de basquetebol mostraram que estes apresentam ritmo circadiano de cortisol normal, e respondem ao estímulo de estresse representado pelo jogo. Além disso, a abordagem metodológica utilizada permitiu o cálculo da área sob a curva que evidenciou a diminuição gradativa da resposta de estresse ao longo do campeonato. A determinação do cortisol salivar também permitiu investigar os índices de estresse em vestibulandos e indicou que estes apresentam altos índices de estresse ao longo do ano, os quais estão associados a sintomas de depressão e ansiedade. Estes sintomas aumentam no final do ano, antes do período de exames e nos meses em que os estudantes fazem as inscrições para os exames. Este contínuo estado de estresse, durante todo o ano, correlacionou-se inversamente com aprovações no vestibular. Em indivíduos de baixo *status* sócio-econômico, as altas concentrações de cortisol correlacionaram-se com altos índices de morbidade psiquiátrica.

Concluindo, sugerimos que cortisol é um marcador fidedigno para identificar a resposta de estresse, e que, quando este é analisado em amostras salivares seriadas é possível melhor interpretação dos resultados.

Salientamos, no entanto, que a concentração salivar de cortisol apresenta grande variação entre indivíduos e em um mesmo indivíduo em dias diferentes e são freqüentes os problemas de aderência ao protocolo de coleta, o que pode comprometer a interpretação dos resultados. A determinação da concentração salivar de outros esteróides considerados hormônios da recuperação, tais como testosterona, dehidroepiandrosterona (DHEA) e corticosterona poderão auxiliar na interpretação dos dados de resposta de estresse, uma vez que estes apresentam melhor estabilidade.

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Anexo 1 -

UNICAMP

FACULDADE DE CIÊNCIAS MÉDICAS
COMITÊ DE ÉTICA EM PESQUISA
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CEP, 18/02/03
(Grupo III)

PARECER PROJETO: N° 604/2002

I-IDENTIFICAÇÃO:

PROJETO: **"INFLUÊNCIA DO ESTRESSE EM ESTUDANTES PRÉ-VESTIUBULANDOS NOS CURSOS DE MAIOR DEMANDA, ENVOLVENDO AS TRÊS ÁREAS DE CONHECIMENTO".**
PESQUISADOR RESPONSÁVEL: Regina Célia Spadari-Bratfisch
INSTITUIÇÃO: Depto de Fisiologia e Biofisica/Instituto de Biologia/UNICAMP
APRESENTAÇÃO AO CEP: 19/12/2002

II - OBJETIVOS

Avaliar o grau de estresse em alunos que buscam uma vaga em cursos concorridos nas tres áreas de conhecimento;Comparar os resultados nos exames simulados realizados por tais alunos durante o ano letivo com os resultados obtidos pelos mesmos alunos nos exames oficiais;Verificar se há correlação entre o nível de estresse e o desempenho nos exames;Verificar se há correlação entre a dificuldade de ingresso (através da comparação do índice: candidatos por vaga) e o nível de estresse.

III - SUMÁRIO

O projeto trabalhará com o jovem que é vestibulando, avaliando os níveis de estresse por que passa neste período, principalmente em cursos de grande competitividade, como os de Medicina, Direito e Engenharia. Acredita-se que neste tempo, a fonte de estresse perdura por pelo menos um ano. No ano de 2001 o estresse foi considerado pela OMS, como um dos principais fatores de risco para inúmeras patologias. O estudo visa avaliar as concentrações salivares de cortisol em jovens que estejam pleiteando uma vaga nestes cursos, já mencionados, bem como levantar o estado emocional deste jovem, através de questionários. Estes parâmetros serão avaliados por meio de correlação com o grau de dificuldade de ingresso caracterizado pela razão entre o número de candidatos e número de vagas. Os sujeitos serão estudantes de colegial e cursinhos, com idade de 18 a 25 anos, de ambos os sexos. A amostra será de 50 voluntários e o financiamento do projeto será pela FAPESP com bolsa de Iniciação Científica.

iV - COMENTÁRIOS DOS RELATORES

O projeto pretende com os resultados obtidos, iniciar um trabalho junto aos alunos com o objetivo de ensiná-los a controlar e conviver com situações de estresse, minimizando

seus efeitos danosos sobre o organismo. Os resultados deste trabalho poderão fundamentar uma discussão sobre o modo como atualmente os jovens brasileiros tem acesso à educação superior de alto nível – o exame vestibular, caso se confirme por meio de parâmetros fisiológicos e testes psicológicos, os elevados índices de estresse que este processo causa.
Termo de Consentimento de acordo.

V - PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP, após acatar os pareceres dos membros-relatores previamente designados para o presente caso e atendendo todos os dispositivos das Resoluções 196/96 e 251/97, bem como ter aprovado o Termo do Consentimento Livre e Esclarecido, assim como todos os anexos incluídos na Pesquisa, resolve aprovar sem restrições o Protocolo de Pesquisa supracitado.

VI - INFORMAÇÕES COMPLEMENTARES

O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 196/96 – Item IV.1.f) e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado (Item IV.2.d).

Pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou (Res. CNS Item III.1.z), exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade do regime oferecido a um dos grupos de pesquisa (Item V.3.). O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS Item V.4.). É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projeto do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial (Res. 251/97, Item III.2.e).

Relatórios parciais e final devem ser apresentados ao CEP, de acordo com os prazos estabelecidos na Resolução CNS-MS 196/96.

VII - DATA DA REUNIÃO

Homologado na II Reunião Ordinária do CEP/FCM, em 18 de fevereiro de 2003.

Prof. Dr. Sebastião Araújo
PRESIDENTE do COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP

Anexo 2 -

UNIFESP
PAULISTA ESCOLA MEDICINA

Universidade Federal de São Paulo
Escola Paulista de Medicina

Comitê de Ética em Pesquisa
Hospital São Paulo

São Paulo, 11 de outubro de 2007.
CEP 0191/07

Ilmo(a). Sr(a).
Pesquisador(a) REGINA CELIA SPADARI
Co-Investigadores: Marcia Carvalho Garcia
Disciplina/Departamento: Ciências da Saúde/Fisiologia da Universidade Federal de São Paulo/Hospital São Paulo
Patrocinador: Recursos Próprios.

PARECER DO COMITÊ DE ÉTICA INSTITUCIONAL

Ref: Projeto de pesquisa intitulado: **"Marcadores hormonais de estresse em atletas de elite de cinco modalidades esportivas".**

CARACTERÍSTICA PRINCIPAL DO ESTUDO: estudo coorte.
RISCOS ADICIONAIS PARA O PACIENTE: sem risco, desconforto mínimo.
OBJETIVOS: Determinar as concentrações dos hormônios marcadores de estresse em atletas de cinco modalidades esportivas, durante a temporada de treinos e de competições, correlacionando-as com o desempenho físico, resultados obtidos em competições e índice de estresse percebido.
RESUMO: Este estudo pretende identificar marcadores de estresse e de recuperação em atletas de cinco modalidades esportivas distintas: basquetebol, natação, thriathlon, tenis e badminton. Serão realizadas coletas de saliva de atletas para quantificar as concentrações de cortisol, corticosterona, alfa-amilase, DHEA-S e testosterona. As coletas serão realizadas entre as fases distintas do treinamento, bem como durante o campeonato mais importante do ano. Participarão do estudo 15 atletas de natação, 12 de basquetebol, 10 de triathlon, 32 de tenis e 20 de badminton.A faixa etária varia de 14 a 35 anos..
FUNDAMENTOS E RACIONAL: Agentes estressores geram respostas orgânicas, ativando complexos sistemas fisiológicos e comportamentais, que resultam em adaptação ou mal-adaptação. A resposta de um atleta a agentes estressores pode resultar em queda, manutenção ou melhora no seu desempenho..
MATERIAL E MÉTODO: Estão descritos os procedimentos e parâmetros a serem avaliados.
TCLE: .
DETALHAMENTO FINANCEIRO: FAPESP - R$ 157 140,00.
CRONOGRAMA: 24 meses.
OBJETIVO ACADÊMICO: doutorado.
ENTREGA DE RELATÓRIOS PARCIAIS AO CEP PREVISTOS PARA: **10/10/2008** e **10/10/2009**.

O Comitê de Ética em Pesquisa da Universidade Federal de São Paulo/Hospital São Paulo **ANALISOU** e **APROVOU** o projeto de pesquisa referenciado.

Rua Botucatu, 572 - 1º andar – conj. 14 - CEP 04023-062 - São Paulo / Brasil
Tel.: (011) 5571-1062 - 5539.7162

1. Comunicar toda e qualquer alteração do projeto e termo de consentimento livre e esclarecido. Nestas circunstâncias a inclusão de pacientes deve ser temporariamente interrompida até a resposta do Comitê, após análise das mudanças propostas.
2. Comunicar imediatamente ao Comitê qualquer evento adverso ocorrido durante o desenvolvimento do estudo.
3. Os dados individuais de todas as etapas da pesquisa devem ser mantidos em local seguro por 5 anos para possível auditoria dos órgãos competentes.

Atenciosamente,

Prof. Dr. José Osmar Medina Pestana
Coordenador do Comitê de Ética em Pesquisa da
Universidade Federal de São Paulo/ Hospital São Paulo

CEP 0191/07