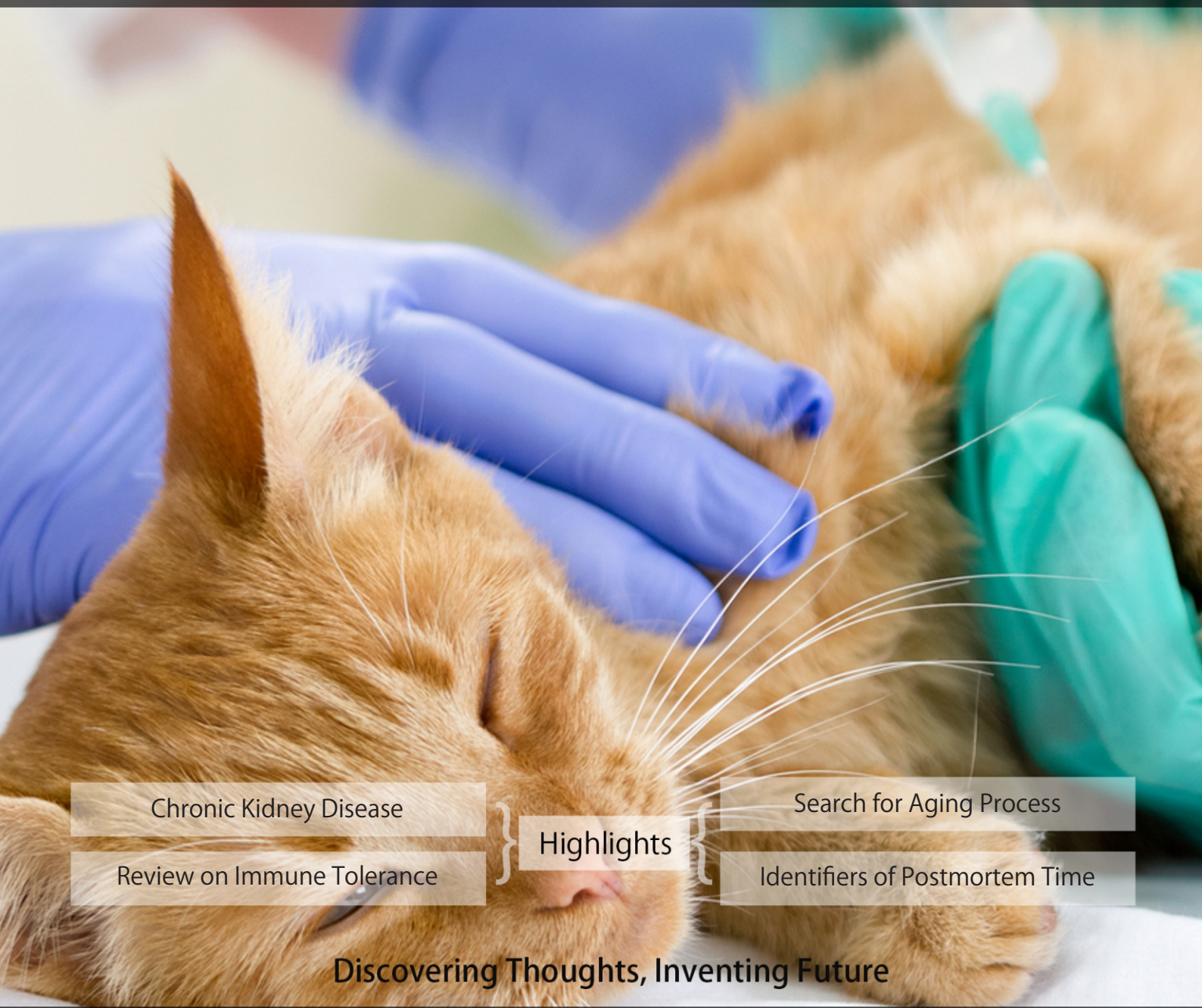


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Chronic Kidney Disease

Review on Immune Tolerance

Highlights

Search for Aging Process

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Oxidative Stress in Dogs in Different Stages of Chronic Kidney Disease

By André B. Galvão, Marileda B. Carvalho, Luciane G. Batalhão, Juliana C.B. Silva, Marcelo Batalhão & Evelin C. Carnio

Abstract- The objective of this study to quantify oxidative stress in dogs with naturally acquired chronic kidney disease (CKD), considering the four stages of disease progression, using as markers reactive substances to thiobarbituric acid. Five groups of dogs, all aged between four to 18 years old. Were studied the control group was composed of healthy animals (CG, n = 17), CKD stage 1 group (CKD-1, n = 12), CKD stage 2 group (CKD-2, n = 10), CKD stage 3 group (CKD-3, n = 13) and CKD stage 4 group (CKD-4, n = 10). Oxidative stress was measured by antioxidant power, using as markers reactive substances to thiobarbituric acid. Data (means of duplicates) were submitted to analysis of variance (One-way ANOVA) nonparametric (Kruskal-Wallis) ($\alpha = 0.05$). The results were expressed as mean \pm standard error of the mean. The serum creatinine values guided the classification of patients as CG, CKD-1, CKD-2, CKD-3 and CKD-4 and were 1.02 ± 0.02 mg/dL, 1.06 ± 0.05 mg/dL, 1.80 ± 0.03 mg/dL, 3.39 ± 0.21 mg/dL and 6.00 ± 0.28 mg/dL, respectively. The results related to antioxidant power (GC) 0.032 ± 0.002 mmol/ μ L, (CKD-1) 0.030 ± 0.002 nmol/ μ L, (CKD-2) 0.028 ± 0.003 nmol/ μ L, (CKD-3) 0.027 ± 0.003 nmol/ μ L and (CKD-4) 0.025 ± 0.002 nmol/ μ L.

Keywords: creatinine, hypertension, proteinuria.

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Oxidative Stress in Dogs in Different Stages of Chronic Kidney Disease

André B. Galvão ^α, Marileda B. Carvalho ^ο, Luciane G. Batalhão ^ρ, Juliana C.B. Silva ^ω, Marcelo Batalhão [¥] & Evelin C. Carnio [§]

Abstract- The objective of this study to quantify oxidative stress in dogs with naturally acquired chronic kidney disease (CKD), considering the four stages of disease progression, using as markers reactive substances to thiobarbituric acid. Five groups of dogs, all aged between four to 18 years old. Were studied the control group was composed of healthy animals (CG, n = 17), CKD stage 1 group (CKD-1, n = 12), CKD stage 2 group (CKD-2, n = 10), CKD stage 3 group (CKD-3, n = 13) and CKD stage 4 group (CKD-4, n = 10). Oxidative stress was measured by antioxidant power, using as markers reactive substances to thiobarbituric acid. Data (means of duplicates) were submitted to analysis of variance (One-way ANOVA) nonparametric (Kruskal-Wallis) ($\alpha = 0.05$). The results were expressed as mean \pm standard error of the mean. The serum creatinine values guided the classification of patients as CG, CKD-1, CKD-2, CKD-3 and CKD-4 and were 1.02 \pm 0.02 mg/dL, 1.06 \pm 0.05 mg/dL, 1.80 \pm 0.03 mg/dL, 3.39 \pm 0.21 mg/dL and 6.00 \pm 0.28 mg/dL, respectively. The results related to antioxidant power (GC) 0.032 \pm 0.002 mmol/ μ L, (CKD-1) 0.030 \pm 0.002 nmol/ μ L, (CKD-2) 0.028 \pm 0.003 nmol/ μ L, (CKD-3) 0.027 \pm 0.003 nmol/ μ L and (CKD-4) 0.025 \pm 0.002 nmol/ μ L. In conclusion dogs with naturally acquired CKD, clinically stable, without therapeutic intervention, presented an increased lipid peroxidation, the intensity is independent of the stage of the disease.

Keywords: creatinine, hypertension, proteinuria.

Estresse oxidativo em cães nos diferentes estádios da doença renal crônica

Resumo- Objetiva- se com este estudo quantificar o estresse oxidativo em cães com doença renal crônica (DRC) naturalmente adquirida, considerando os quatro estágios de progressão da doença, utilizando como marcadores as substâncias reativas ao ácido tiobarbitúrico. Foram estudados cinco grupos de cães, com idade variando entre quatro a 18 anos, compreendendo o grupo controle, composto por animais sadios (controle, n=17), grupo com DRC estágio 1 (DRC-1, n=12), grupo com DRC estágio 2 (DRC-2, n=10), grupo com DRC estágio 3 (DRC-3, n=13) e grupo com DRC estágio 4 (DRC-4, n=10). Os cães com DRC estavam com

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o quadro clínico estável e sem receber qualquer tipo de tratamento. O estresse oxidativo foi avaliado pelo poder antioxidante, estimado pelo delta, por meio da mensuração das substâncias reativas ao ácido tiobarbitúrico. Os dados obtidos (médias das duplicatas) foram submetidos à análise de variância (One-way ANOVA) não paramétrica (Kruskal-Wallis) ($\alpha = 0.05$). Os resultados expressos como média \pm erro padrão da média. Os valores de creatinina sérica, que definiram a classificação dos pacientes do grupo controle, DRC-1, DRC-2, DRC-3 e DRC-4 foram 1,02 \pm 0,02 mg/dL; 1,06 \pm 0,05 mg/dL; 1,80 \pm 0,03 mg/dL; 3,39 \pm 0,21 mg/dL e 6,00 \pm 0,28 mg/dL, respectivamente. Os resultados relativos ao delta foram (Controle) 0,032 \pm 0,002 nmol/ μ L, (DRC-1) 0,030 \pm 0,002 nmol/ μ L, (DRC-2) 0,028 \pm 0,003 nmol/ μ L, (DRC-3) 0,027 \pm 0,001 nmol/ μ L e (DRC-4) 0,025 \pm 0,002 nmol/ μ L. Concluiu-se que cães com DRC naturalmente adquirida que se encontram clinicamente estáveis e sem intervenção terapêutica tende diminuir o poder antioxidante.

Palavras-Chave: creatinina, hipertensão, proteinúria.

I. INTRODUÇÃO

O desequilíbrio entre compostos oxidantes e antioxidante compreende na instalação do estresse oxidativo. Com este desequilíbrio, favorece a geração e acúmulo de espécies reativas de oxigênio (ERO) (Barbosa et al., 2010).

O paciente com transtornos renais, geralmente, apresenta-se mal nutrido, com carências em reservas de vitaminas e minerais, o que diminui os mecanismos de defesa antioxidante e favorece a instalação do estresse oxidativo (Locatelli et al., 2003). Estas condições facilitam a instalação do estresse oxidativo no paciente com doença renal crônica (DRC), bem como estão diretamente com as consequências no comprometimento de outros sistemas (Urso e Caimi, 2011).

Ao longo do curso da DRC em cães, o paciente pode passar por diferentes estádios em função do grau de comprometimento e severidade da enfermidade. De acordo com a classificação estabelecida pela "International Renal Interest Society" (Iris, 2013), no que concerne à condição renal, cães podem ser classificados como em pacientes sob risco de desenvolver DRC; e pacientes com diagnóstico estabelecido de DRC que podem ser categorizados em quatro estádios distintos de acordo com dados clínicos e laboratoriais. No estágio 1, o paciente não é azotêmico (creatinina sérica < 1,4 mg/dL); o estágio 2 é

caracterizado por azotemia renal leve (creatinina sérica $1,4 \geq 2,0$ mg/dL) e os sinais clínicos geralmente são leves ou ausentes; no estágio 3 existe azotemia renal moderada (creatinina sérica $2,1 \geq 5,0$ mg/dL) e os sinais clínicos podem estar presentes; e no estágio 4, o paciente apresenta azotemia renal severa (creatinina sérica $> 5,0$ mg/dL) e os sinais clínicos geralmente estão presentes.

Em condições de comprometimento da função renal associada com o estresse oxidativo, ocorre a elevação de biomarcadores no sangue, tal como o malondialdeído MDA (Cachoeiro et al., 2008). O MDA é um produto secundário da peroxidação lipídica, sendo considerado um candidato potencial para ser escolhido como um biomarcador geral do dano oxidativo no soro e no plasma (Vasconcelos et al., 2007).

O MDA foi o foco de atenção da peroxidação lipídica durante muitos anos, pelo fato de poder ser medido livre, utilizando-se o ácido tiobarbitúrico (TBA) (Vasconcelos et al., 2007). Assim, objetiva-se com este estudo quantificar o estresse oxidativo em cães com DRC considerando os quatro estágios de progressão da DRC, utilizando como marcadores as substâncias reativas ao ácido tiobarbitúrico.

II. MATERIAL E MÉTODOS

Foram estudados cinco grupos de cães, compreendendo o grupo controle, composto por animais saudáveis (controle, $n=17$), grupo com DRC estágio 1 (DRC-1, $n=12$), grupo com DRC estágio 2 (DRC-2, $n=10$), grupo com DRC estágio 3 (DRC-3, $n=13$) e grupo com DRC estágio 4 (DRC-4, $n=10$). Os cães com DRC estavam com o quadro clínico estável e sem receber qualquer tipo de tratamento. Os cães avaliados foram provenientes do canil do GPNUV e do atendimento do Serviço de Nefrologia e Urologia Veterinária (SNUV) do Departamento de Clínica e Cirurgia Veterinária-Unesp-Câmpus de Jaboticabal-SP.

Para a formação dos grupos, os cães foram avaliados clínica e laboratorialmente, de acordo com a abordagem semiológica descrita por Carvalho (2014). Para compor o grupo controle os cães deviam ser adultos saudáveis, sem restrição de sexo ou raça. Para compor os grupos de animais doentes os cães deveriam ser adultos, sem restrição de sexo ou raça e apresentar sinais clínicos e laboratoriais de DRC, nos estágios 1, 2, 3 ou 4, em condição clínica estável. Os motivos de exclusão compreenderam existência de urolitíase, obstrução urinária, infecção ou neoplasia de trato urinário, crise urêmica, comorbidades, necessidade de tratamento imediato, pacientes já em tratamento farmacológico, alimentar ou de reposição.

A inclusão de pacientes do SNUV foi feita sob anuência dos proprietários que se dispuseram a trazer o seu cão para duas avaliações respeitando o intervalo de 24 horas de acordo com o preconizado pela Iris

(2013). Para tanto, os proprietários foram devidamente esclarecidos sobre o desenho experimental.

Todos os animais doentes foram devidamente assistidos e foram iniciadas as intervenções terapêuticas, recomendadas para cada caso, após a segunda coleta de amostras.

O protocolo experimental do presente trabalho foi previamente aprovado, pela Comissão de Ética no uso de Animais (CEUA) conforme processo n.º 013690/11.

Sendo elegíveis para compor os grupos, os pacientes saudáveis ou com DRC, foram submetidos a duas sessões de avaliação (exame clínico de rotina e coletas de amostras de sangue e urina) com intervalo de 24 horas.

A avaliação dos animais incluiu exame físico de rotina, mensuração da pressão arterial sistólica, hemograma, urinálise, avaliação da excreção urinária de proteína, análise do perfil bioquímico e eletrolítico sérico (ureia, creatinina, proteína total, albumina, alanina aminotransferase, fosfatase alcalina, sódio, potássio, fósforo, cálcio total e cálcio iônico), e os ensaios para mensuração de indicadores de estresse oxidativo (concentrações das substâncias reativas ao ácido tiobarbitúrico).

Para a determinação da pressão arterial sistólica, foi utilizado o aparelho doppler vascular¹, dotado de módulo de coleta não-invasiva. Os animais foram posicionados em decúbito lateral direito e o manguito² foi colocado no membro torácico esquerdo, entre o olecrano e o carpo. Os manguitos utilizados apresentaram aproximadamente 40% da circunferência do local em que foram colocados no membro torácico. Foram realizadas sete determinações e os valores limítrofes superiores e inferiores descartados para a obtenção de uma média mais acurada (Carvalho, 2014). Depois de estabelecida as médias os valores foram classificados utilizando os critérios estabelecidos pela Iris (2013).

As contagens globais de eritrócitos, leucócitos, e plaquetas, bem como a taxa de hemoglobina e hematócrito, VGM e CHGM foram obtidas com o auxílio de um contador automático³. As contagens diferenciais de leucócitos foram realizadas em esfregaços sanguíneos corados com mistura de Metanol, May-Gruwald e Giemsa. As amostras foram processadas no período máximo de uma hora após a coleta.

A proteinúria foi avaliada por meio da determinação da razão proteína/creatinina da urina (U-P/C), a partir dos valores de concentração de creatinina e de proteína obtidas na mesma amostra de urina. Para a urinálise e avaliação da proteinúria, as amostras de urina foram obtidas por meio de cateterização

¹ Doppler Vascular DV10 Pastilha Microem – Ribeirão Preto – SP.

² Manguito Neonatal dois tubos.

³ COULTER modelo ABC T8.

transmural e analisadas no máximo 30 minutos após a coleta. Para os testes químicos foram utilizadas fitas reagentes⁴.

As amostras de soro foram processadas para determinação de creatinina (método Jaffé modificado), ureia (método enzimático), proteína total (método biureto), albumina (método verde de bromocresol), cálcio total (método da cresoltaleina complexona), e fósforo (método do fosfomolibdato). Nas amostras de urina foram dosadas creatinina (método Jaffé modificado) e proteína total (método do vermelho de pirogalol). Todas as análises bioquímicas foram feitas com os conjuntos de reagentes do sistema Labtest⁵ para diagnóstico. Para as leituras foi empregado espectrofotômetro⁶ semi-automático.

As concentrações séricas de sódio, potássio e cálcio iônico foram feitas pelo método de eletrodo íon-seletivo⁷.

Para analisar os indicadores de estresse oxidativo, os ensaios foram conduzidos em duplicata das amostras. Para determinação das substâncias reativas ao ácido tiobarbitúrico foram realizados ensaios únicos, para evitar erros entre ensaios.

A extensão da degradação da desoxirribose por radicais hidróxi (OH⁻) gerados neste sistema foi mensurada pelo ensaio do ácido tiobarbitúrico (TBA) descrito por Payá et al., (1992). Alíquotas das amostras foram diluídas em solução tampão Tris-ácido cítrico mantendo-se o padrão 100 µL de soro. Das duas alíquotas de soro a primeira foi adicionada a 2mL da solução de TBA (15% de Ácido Tricloroacético, 0,275% de Ácido Tiobarbitúrico e 0,25 M de Ácido Clorídrico) para a obtenção da peroxidação lipídica espontânea. Em seguida, os tubos foram aquecidos a 100°C por 15 minutos e, então, foram resfriados e centrifugados por 15 minutos a 1200g para formação de precipitado. O sobrenadante foi analisado ao espectrofotômetro. O cromógeno desenvolvido foi identificado por meio de leituras de absorvância em um comprimento de onda fixo de 532nm. A segunda alíquota foi adicionada a 2mL de solução de TBA-SG (15% de Ácido Tricloroacético, 0,375% de Ácido Tiobarbitúrico, 0,25M de Ácido Clorídrico, 0,24mM de Cloreto de Ferro e 50 µM de Hidroxitoluenobutilado) para obtenção da peroxidação lipídica induzida. Esse procedimento, denominado sistema gerador, tem a mesma função do sistema gerador para os radicais O₂⁻ e H₂O₂. Os resultados da mensuração de peroxidação lipídica foram expressos em nmol/µL.

A solução de TBA quantifica a peroxidação lipídica espontânea, enquanto a solução TBA-SG

quantifica a peroxidação lipídica induzida ou catalizada. Na diferença entre TBA-SG e TBA, se obtém o delta, que indica o poder antioxidante (Buege e Aust, 1978).

O experimento seguiu delineamento em blocos casualizados com distribuição dos animais de acordo com o diagnóstico. Os resultados foram submetidos à análise de variância (One-way ANOVA) não paramétrica (Kruskal-Wallis), considerando o fator grupo, seguida de teste de Dunn de comparação múltipla das médias ($\alpha = 0,05$).

As análises foram realizadas pelo programa GraphPad Prisma version 6.02 for Windows, GraphPad Software, La Jolla California USA.

III. RESULTADOS

O perfil renal dos 17 cães saudáveis e dos 45 cães com DRC que foram distribuídos em quatro grupos em função do estágio da doença encontram-se na Tabela 1 os resultados (média±erro padrão da média e comparação múltipla das médias) das concentrações séricas de creatinina (Scr), densidade urinária (DU), razão proteína/creatinina urinária (U-P/C) e pressão arterial sistólica (PAS) alicerçam a classificação.

As médias de Scr não diferiram significativamente entre si quando comparados os grupos Controle e DRC-1, mas houve diferença significativa entre estes e os grupos DRC-2, DRC-3 e DRC-4. Também foi constatado aumento significativo progressivo das médias de Scr relacionado com os estágios da doença. O comprometimento da capacidade de concentrar a urina, estimada pela DU, foi evidenciado por diminuição gradativa das médias, que diferiram significativamente nos grupos DRC-2, DRC-3 e DRC-4 em relação à do Controle (Tabela 1).

Os dados relativos à U-P/C resultaram em médias significativamente maiores que a do Controle, nos grupos DRC-1, DRC-3 e DRC-4, mas não no grupo DRC-2. O mesmo quadro foi observado em relação à PAS, cuja média do grupo DRC-2 não diferiu significativamente das do Controle e DRC-1, embora as médias dos grupos DRC-1, DRC-3 e DRC-4 tenham sido significativamente maiores que a do Controle (Tabela 1).

⁴ COMBUR 10 Test UX® - Boehringer Mannheim S.A. – Buenos Aires – Argentina.

⁵ LABTEST – Labtest Diagnóstica S.A., Lagoa Santa – MG.

⁶ LABQUEST-LABTEST – Labest Diagnóstica S.A., Lagoa Santa – MG.

⁷ ISELAB – Drake – São José do Rio Preto, SP, Brasil.

Tabela 1: Resultados (média \pm erro padrão) referentes aos parâmetros considerados como critérios para inclusão dos animais no grupo de cães saudáveis (Controle; n=17) ou nos grupos de cães com doença renal crônica nos estágios 1 (DRC-1; n=12), 2 (DRC-2; n=10), 3 (DRC-3; n=13), e 4 (DRC-4; n=10). Os dados foram obtidos em dois momentos (repetição) com intervalo de 24 horas

Variável	Grupos avaliados				
	Controle	DRC-1	DRC-2	DRC-3	DRC-4
Scr (mg/dL)	1,02 \pm 0,02c	1,07 \pm 0,04c	1,81 \pm 0,03b	3,40 \pm 0,15ab	6,00 \pm 0,20a
DU	1,034 \pm 0,00a	1,023 \pm 0,00ac	1,017 \pm 0,00bc	1,013 \pm 0,00b	1,012 \pm 0,00b
U-P/C	0,13 \pm 0,01c	1,14 \pm 0,12b	0,59 \pm 0,16c	1,96 \pm 0,25ab	1,53 \pm 0,25ab
PAS (mmHg)	137,9 \pm 1,9b	181,3 \pm 6,1a	159,0 \pm 6,1ab	186,9 \pm 6,1a	180,0 \pm 7,3a

Médias, na mesma linha, seguidas de pelo menos uma letra em comum não diferem estatisticamente entre si (Teste de Dunn $\alpha=0,05$). Scr = Creatinina Sérica; DU = Densidade Urinária; U-P/C = Razão Proteína/Creatinina Da Urina, PAS = Pressão Arterial Sistólica.

Além da Scr, utilizada como critério para composição dos grupos, foram avaliados diversos outros parâmetros da bioquímica sérica dos cães estudados (Tabela 2). A média de concentração sérica de ureia (Sureia) do grupo DRC-2 foi significativamente maior do que a do Controle e as médias dos grupos DRC-3 e DRC-4 foram significativamente maiores em relação às dos demais. Um quadro semelhante foi observado com as médias de concentração sérica de fósforo (SP).

As médias de concentrações séricas de cálcio total (SCat) dos grupos DRC-3 e DRC-4 foram significativamente maiores que a do Controle e somente a média do DRC-4 foi significativamente maior que as dos grupos DRC-1 e DRC-2. Contudo, as concentrações séricas de cálcio ionizados (SCai) não variaram significativamente entre os grupos.

Quanto às concentrações séricas de sódio (SNa) e de potássio (SK), as diferenças mais marcantes foram observadas no grupo DRC-2 que apresentou média de SNa significativamente maior que as dos grupos DRC-1 e DRC-4 e a média de SK significativamente menor que as observadas nos outros quatro grupos.

As médias de concentrações séricas de proteína total (SPT) e de albumina (SAIb) dos grupos DRC-3 e DRC-4 foram significativamente menores que as respectivas médias do grupo Controle.

Tabela 2: Resultados (média \pm erro padrão) do perfil bioquímico sérico de cães saudáveis (Controle; n=17) e de cães com doença renal crônica nos estágios 1 (DRC-1; n=12), 2 (DRC-2; n=10), 3 (DRC-3; n=13), e 4 (DRC-4; n=10). Os dados foram obtidos em dois momentos (repetição) com intervalo de 24 horas.

Variável	Grupos avaliados				
	Controle	DRC-1	DRC-2	DRC-3	DRC-4
Sureia (mg/dL)	31,18 \pm 1,73c	51,85 \pm 5,33cd	80,17 \pm 11,22bd	177,30 \pm 14,73a	223,10 \pm 11,23a
SP (mg/dL)	4,16 \pm 0,16c	3,90 \pm 0,30c	5,28 \pm 0,36bc	6,41 \pm 0,46ab	8,26 \pm 0,73a
SCat (mg/dL)	10,17 \pm 0,19c	10,37 \pm 0,18bc	10,28 \pm 0,15bc	10,97 \pm 0,20ab	11,52 \pm 0,30a
SCai (mg/dL)	1,07 \pm 0,03a	1,16 \pm 0,02a	1,10 \pm 0,05a	1,03 \pm 0,05a	1,08 \pm 0,06a
SNa (mEq/L)	148,10 \pm 0,42ab	146,30 \pm 1,00b	150,60 \pm 0,71a	148,26 \pm 1,14ab	146,70 \pm 0,87b
SK (mEq/L)	4,70 \pm 0,07ab	4,60 \pm 0,10b	4,20 \pm 0,09c	5,15 \pm 0,11a	5,02 \pm 0,16ab

Spt (g/dL)	7,60 ± 0,09a	7,29±0,11ab	7,43 ± 0,17ab	6,99 ± 0,16b	6,88 ± 0,26b
SAIb (g/dL)	3,33 ± 0,08a	3,18 ± 0,11ab	3,28 ± 0,09ac	2,75 ± 0,13b	2,86 ± 0,12bc
SALT (mg/dL)	55,39 ± 3,22a	51,67±4,44a	52,28 ± 4,33a	61,04 ± 3,37a	44,43 ± 4,02a
SFA (mg/dL)	63,23±6,66a	57,34±5,47a	71,20 ± 8,54a	70,31 ± 7,50a	49,40 ± 4,36a

Médias, na mesma linha, seguidas de pelo menos uma letra em comum não diferem estatisticamente entre si (Teste de Dunn; $\alpha=0,05$). Sureia = Ureia Sérica; SP = Fósforo Sérico; SCat = Cálcio Total Sérico; SCai = Cálcio Ionizado Sérico; SNa = Sódio Sérico; SK = Potássio Sérico; Spt = Proteína Total Sérica; SAlb = Albumina Sérica; SALT = Alanina Aminotransferase Sérica; SFA = Fosfatase Alcalina Sérica;

Tabela 3: Resultados (média ± erro padrão) do perfil eritrocitário, plaquetário e leucocitário de cães saudáveis (Controle; n=17) e de cães com doença renal crônica nos estágios 1 (DRC-1; n=12), 2 (DRC-2; n=10), 3 (DRC-3; n=13) e 4 (DRC-4; n=10). Os dados foram obtidos em dois momentos (repetição) com intervalo de 24 horas

Variável	Grupos Avaliados				
	Controle	DRC-1	DRC-2	DRC-3	DRC-4
He (x10 ⁶ /μL)	7,31 ± 0,16a	6,93 ± 0,33a	6,17 ± 0,22ab	4,87 ± 0,29bc	4,35 ± 0,24c
Hb (g/dL)	17,59 ± 0,39a	15,54 ± 0,75a	14,97 ± 0,56ab	11,98 ± 0,80bc	10,54 ± 0,63c
Ht (%)	53,66 ± 1,24a	48,21 ± 2,30ab	43,85 ± 1,65bc	36,54 ± 1,97cd	31,88 ± 1,81d
VGM (fL)	73,18 ± 0,57a	71,79 ± 0,78a	73,50 ± 0,67a	73,85 ± 0,46a	72,00 ± 0,77a
CHGM (g/dL)	32,56 ± 0,42a	31,83 ± 0,62a	33,40 ± 0,54a	33,74 ± 0,47a	32,19 ± 0,89a
Plaq (x10 ³ /μL)	328,41±12,71a	315,33±18,55a	324,60±23,71a	385,30±24,81a	336,10±17,19a
Le (x10 ³ /μL)	8,57 ± 0,36b	8,36 ± 0,49b	11,82 ± 0,80a	8,98 ± 0,64ab	10,48 ± 1,00ab
Ns (x10 ³ /μL)	6,11 ± 0,30b	6,02 ± 0,41b	9,06 ± 0,70a	6,26 ± 0,45b	7,30 ± 0,69ab
Linf (x10 ³ /μL)	1,60 ± 0,12a	1,49 ± 0,17a	1,90 ± 0,21a	1,85 ± 0,22a	1,46 ± 0,10a

Médias, na mesma linha, seguidas de pelo menos uma letra em comum não diferem estatisticamente entre si (Teste de Dunn; $\alpha=0,05$). He = Hemácias; Hb = Hemoglobina; Ht = Hematócrito; VGM = volume globular médio; CHGM = concentração de hemoglobina globular média; Plaq = Plaquetas; Le = Leucócitos totais; Ns = Neutrófilos segmentados; Linf = Linfócitos.

Quanto a avaliação do estresse oxidativo, a média da lipoperoxidação espontânea (TBA) do grupo DRC-3 foi significativamente maior do que a do grupo Controle. As demais médias (DRC-1, DRC-2 e DRC-4) não diferiram significativamente entre si ou em relação

às médias do Controle e do DRC-3 (Tabela 4). As médias da lipoperoxidação induzida (TBASG) não diferiram significativamente entre si (Tabela 4). As médias do poder antioxidante (Delta TBASG-TBA) não diferiram significativamente entre si (Tabela 4).

Tabela 4: Dados (média ± erro padrão da média) das concentrações séricas das substâncias reativas ao ácido tiobarbitúrico, referente à lipoperoxidação induzida TBASG, lipoperoxidação espontânea – TBA, delta TBASG-TBA de cães saudáveis (Controle; n=17), e cães com doença renal crônica nos estágios 1 (DRC-1; n=12), 2 (DRC-2; n=10), 3 (DRC-3; n=13) e 4 (DRC-4; n=10). Os dados foram obtidos em dois momentos (repetição) com intervalo de 24 horas

Variável	Grupos Avaliados				
	Controle	DRC-1	DRC-2	DRC-3	DRC-4
TBASG (nmol/μL)	0,057±0,003a	0,060±0,002a	0,053±0,002a	0,057±0,003a	0,055±0,003a
TBA (nmol/μL)	0,025±0,001b	0,030±0,001ab	0,030±0,001ab	0,031±0,001a	0,030±0,002ab
Delta (nmol/μL)	0,032±0,002a	0,030±0,002a	0,028±0,003a	0,027±0,003a	0,025±0,002a

Médias, na mesma linha, seguidas de pelo menos uma letra em comum não diferem estatisticamente entre si (Teste de Dunn; $\alpha=0,05$). TBASG = lipoperoxidação induzida; TBA = lipoperoxidação espontânea; Delta = poder antioxidante (diferença entre TBASG e TBA).

IV. DISCUSSÃO

Depois de estabelecida a DRC, a magnitude da disfunção renal geralmente permanece estável por meses ou declina vagarosamente no decorrer de meses

a anos (Zatz et al., 2012). Segundo Galvão et al. (2013) após a instalação do dano renal no cão ocorrem mudanças estruturais e adaptativas dos néfrons remanescentes, a adaptação inicial decorre em hipertrofia e hipertensão glomerular, na tentativa de

manter a taxa de filtração glomerular (TFG), que promove alterações funcionais.

A disfunção renal é frequentemente associada à condições de desequilíbrio oxidativo. Os diferentes marcadores de tal processo, como o MDA estimado pelo TBA, podem se mostrar elevados em graus variados conforme o grau de progressão da enfermidade e condição clínica do paciente humano (Cachofeiro et al., 2008; Karamouzis et al., 2008). No entanto, Small et al., (2012) descreveram que o TBA para o estudo do estresse oxidativo em amostras séricas de pacientes humanos consiste de uma metodologia inespecífica por ser muito sensível a artefatos. No presente estudo buscou-se estimar a atividade do estresse oxidativo em cães com DRC estágios 1 a 4, utilizando como parte desta avaliação a concentração sérica de TBA e o delta, sendo observado apenas a média do DRC-3 em relação ao TBA significativamente maior quando comparada aos demais grupos estudados. Entretanto, os valores das médias de TBA observados nos grupos Controle, DRC-1, DRC-2 e DRC-4 não diferiram significativamente entre si, mesmo em condições de estadiamento diferente da enfermidade, porém, em se tratando de animais clinicamente estáveis, mesmo com graus variados de disfunção renal, o sistema de defesa antioxidante enzimático, desses pacientes, poderia estar satisfatório, o que pode ter contribuído para que a concentração sérica de TBA não elevasse conforme o estágio da doença. Em relação ao delta as medias dos grupos não diferiram entre si, entretanto, observamos que ocorre uma tendência de diminuição do poder antioxidante conforme o estagiamento da DRC, segundo Scoot (2008) o paciente paciente doente renal crônico apresenta-se mal nutrido, com carências em reserva de vitaminas e minerais, o que diminui os mecanismos de defesa antioxidante, e favorece a instalação do estresse oxidativo.

Segundo Schmid e Schiffel (2010) anemia é um dos achados mais comuns em pacientes com DRC, que predispõe ao estresse oxidativo, pois as hemácias representam o principal componente de defesa antioxidante, por possuírem altas concentrações de enzimas (glutathione), capazes de metabolizar as ERO. A hipóxia tecidual aumenta consideravelmente a produção das ERO e o componente lipídico da membrana eritrocitária está também sujeito à agressão oxidativa. Os produtos desta lipoperoxidação podem induzir o estresse oxidativo intracelular e, na ocorrência de deficiência da defesa do sistema antioxidante, ocorrerá a hemólise. Lustoza (2004) descreveu que o aumento da produção das ERO que ocorre em estados anêmicos crônicos, é seguida da diminuição das defesas antioxidantes do sangue, no presente estudo foi observado uma tendência de diminuição do delta conforme o estagiamento da DRC, adicionalmente, notamos a

diminuição dos parâmetros do perfil eritrocitário conforme o grau de estagiamento da DRC, conforme o valor da média observado de cada grupo com DRC em relação ao grupo Controle. Embora, os pacientes com DRC do presente estudo não apresentassem graus de anemia severa, os achados dos autores supracitados, são semelhantes aos nossos achados. Adicionalmente, quanto ao perfil eritrocitários dos nossos cães, observamos que o VGM e CHGM dos pacientes com DRC, independente do estágio, encontravam-se em valores de normalidade para espécie, mesmo na presença de anemia, segundo Navarro-Garcia (2005) a anemia normocítica normocrômica não regenerativa é comum em pacientes com DRC.

V. CONCLUSÃO

As avaliações relativas às concentrações séricas das substâncias reativas ao ácido tiobarbitúrico revelaram resultados pouco conclusivos, que podem indicar inespecificidade dos métodos empregados ou amostragem insuficiente. Contudo, os resultados sugerem tendência a aumento da peroxidação lipídica espontânea e diminuição do poder antioxidante nos pacientes com DRC.

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Biochemical Identifiers of Postmortem Time Interval on Autopsy of Albino Rats versus Physiological One

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Abstract- Post mortem time interval is still controversial point as no definitive markers estimate successful time since death even there are many studies on human or experimental models investigate physical, biochemical and molecular marker for identification periods of time elapsed after death. After death, no system or organ work in integrity manner, body loss its immune defence and no elimination of breakdown of each cells which could be explain elevation of certain biochemical in serum of dead animal. In this study, we induced brain stem death induced in albino male rat and take heart blood at different time point for analysis of PH, Ast, Alt, lactate dehydrogenase, creatinine, s-fas and tnfa. It was found that PH reduced while serum lactate dehydrogenase was increased in time dependent manner. Notably, s-fas and tnfa increase in dependent manner until last time point. But AST, AIT, BUN and creatinine shown no change except little increase in last time point. On conclusion group of biochemical could be used in estimation of postmortem time interval for each time point.

Keywords: *post mortem time interval, serum biochemicals, LDH, s-fas and tnfa, albino rats.*

GJMR-G Classification: *NLMC Code: QW 70*



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I. INTRODUCTION

The accurate detection of time elapsed since death is one of the most challenges in forensic medicine. there are many methods used to detect the postmortem interval, including physical, chemical and genetic methods (1).

In forensic medicine, the estimation of the time elapsed since death is important due to its role in knowing possible criminal cases and help in apply justice and penalties.

The previously known different elements of the postmortem interval can be measurable from 1 day to many years after death. The determination of the postmortem interval is based on the different changes that a cadaver passes after death including physical, chemical, metabolic processes, bacterial processes and the effect of insect activity (2). The estimating the time since death can be detected quantitatively and qualitatively (3).

Determination of the time since death by chemical means based on systematic patho-

physiological alteration and are found to be more benefit since the effect of external conditions is less (4). The Concentrations of hypoxanthine, NADH, ammonia, and formic acid increased with time and these metabolites may be good markers for post-mortem interval (5). Moreover, in the post-mortem period vitreous humour Na⁺, K⁺ and Ca²⁺ concentration has been investigated for many years. Various authors have found the correlation between increasing these cations and post-mortem interval (6). Additionally, the correlation between total and direct bilirubin, uric acid, urea, transferrin, immunoglobulin M (IgM), creatine kinase (CK), aspartate transaminase (AST), iron and calcium increased significantly with with the time of blood putrefaction (7).

The estimation of the time elapsed since death by molecular techniques reported that a time-dependent increase in the mRNA expression of mRNA expression of Fas Ligand (FasL) and phosphatase and tensin homologue deleted on chromosome 10 (PteN) by Quantitative-PCR. A direct linear correlation was found between the mRNA levels of both proteins up until 6 h after death, using a regression analysis (8). Additionally, postmortem serum levels of HMGB1 protein of 90 male Wistar rats preserved at 4, 14 and 24°C since death were estimated by enzyme-linked immunosorbent assay. The serum hmGB1 level showed a time-dependent increase. These observation provide that HMGB1 is related to the postmortem interval in rats up to seven days at 4°C. (9).

The rationale of these study to investigate different biochemicals in serum or liver tissue homogenate enhanced identification of postmortem interval on rats.

II. MATERIALS AND METHODS

a) Animal Protocol

Animal procedures were conducted with the approval of the animal care committee of the ethics Board of the faculty of veterinary medicine mansoura university (EGYPT).

25 male Albino rats (weight, 230-260 g; age were purchased from faculty of pharmacy, mansoura University, EGYPT). They were maintained on a 12-h light/dark cycle with free access to food and water. Brain

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stem death was induced according (10). Dead rat were kept in fixed supine position on temperature for 30 hour post mortem (august 2017).

Blood samples of each 4 rat for each time point were collected from the heart and great vessels at autopsy at 0, 2, 4, 8, 16 and 30 hours postmortem. Samples were centrifuged immediately for 20 min at 5000 rpm. Additionally, blood samples collected from living rat from retroflexus serve as control physiological group and separated for 20 min at 5000 rpm. Serum samples was stored at - 70 liquid nitrogen until measurement.

b) *Quantitative pH and biochemical activity detection*

Blood pH was detected using pH meter (Sigma-Aldrich). Certified calibration buffer standards (4 or 8 pH) (Sigma-Aldrich) were used before each pH analysis. AST, ALT, BuN, creatinine and lactate dehydrogenase were analysed in serum samples.

c) *Concentration of serum FAS ligand and TNFa were detected on by ELISA*

The monoclonal antibodies of rat TNFa (sc52746) and Fas (sc-74540) used for ELISA were used as follows: ELISA plates were coated with rat TNFa and Fas antibody (5 µg/ml) and then incubated overnight at 4°C. The plates were blocked with phosphate-buffered saline (PBS)-3% bovine serum albumin (BSA) for 1 h at room temperature and then incubated with the test

sample for 4 to 5 h at room temperature. The plates were washed and sequentially incubated with biotinylated secondary antibody, avidin-alkaline phosphatase, and substrate. The OD was read at 405 nm by using ELISA reader.

d) *Statistical Analysis*

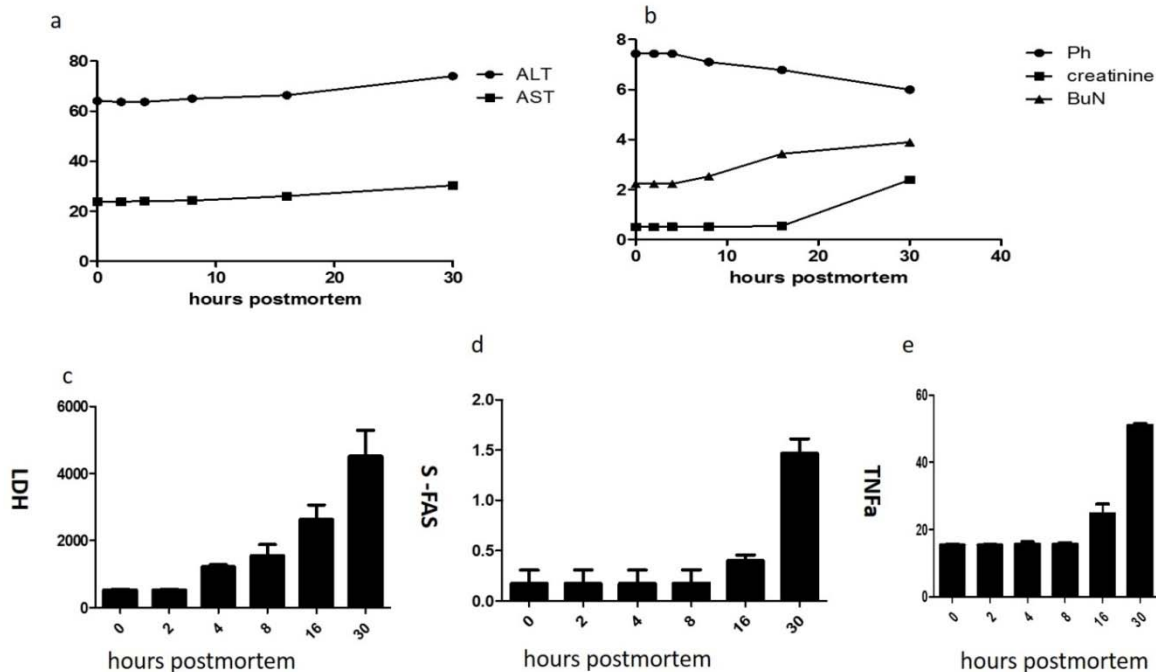
Statistical analysis was carried out using the student's t-test. P<0.05 was considered significant.

III. RESULTS

Post mortem time interval still controversial , to better understand the determinant biochemicals identify time elapsed since death, we induced death in male albino rats and measured different biochemicals in first 30 hours post death at defined times point.

AST, AIT, BUN and creatinine shown no change except little increase in last two successful time points see figure (a, b). Additionally, it was found that PH reduced as lactate escape from tissue while serum lactate dehydrogenase was increased in time dependent manner as leakage from tissue and may be a part of its increase due to hemolysis figure (1 c, d).

Notably, s-fas and tnfa increase in dependent manner until last time point see figure 1(d, e). On conclusion no group of biochemicals could be used in estimation of postmortem time interval for each time point.



Figure

IV. DISCUSSION

Post mortem time interval is still have interest for research from forensic point as no definitive markers estimate successful time since death even there are

many studies on human or experimental models investigate physical, biochemical and molecular marker for identification periods of time elapsed after death. After death, body loss its immune defence , aerobic oxidation and no elimination of breakdown of each cells

which could be explain elevation of certain biochemical parameters in serum of dead animal.

The aim of this study was to investigate metabolic changes that occur in blood post-mortem, with a view towards identifying biochemical markers that have potential for use in determining post-mortem interval. Blood pH and the concentrations of serum lactate dehydrogenase, AST, ALT, creatinine, BUN, TNFa and s-FAS changes post-mortem in rat corpses blood.

Blood is potential material as it remained liquid inside the rat corpses over the 96 hour of observation period due to release of active fibrinolysin enzymes from the vascular wall (11, 12 and 5). Post-mortem blood pH fell from 7.4 to 6.0 within the first 30 hours after death. Blood pH reduced post-mortem due to accumulation of lactic acid which resulted from anaerobic oxidation, blood stagnation in most dependant part and loss of buffer system (13 and 5).

Hepatocyte autolysis result in release of liver enzyme but this only happen when the putrefaction start in the corpse (5 and 7). While stop renal function retain creatinine but not occur immediately after death as Proteolysis did not occur uniformly throughout the body due to the resistance of some proteins such as collagen to breakdown (14). Blood does not contain substances that elevate the creatinine level Serum creatinine correlated significantly with lean mass (15) and so muscle creatinine may entered the blood and elevated the serum creatinine level.

Lactate dehydrogenase was decreased to 131 U/mg 24 hours post mortem on human dental pulp (16). Also a loss of lactate dehydrogenase activity was observed and distinguish between fresh and frozen-thawed fish fillets (17). Notably, lactate and malate dehydrogenases were detected in tissue extracts of human liver kept at 5 different temperatures until 35 days after death. The investigated activities of lactate dehydrogenase were reduced in proportion to time of storage which enabled detection of time elapsed after death (18). As in the current study, serum lactate dehydrogenase activity was increased by prolonged storage of dead rat due to leakage from tissue and also little hemolysis may be a p art of increase level of lactate dehydrogenase.

In the present study, both sfas and tnfa were increased especially at 30 hours after death induction. Increased Fas and FasL immunoreactivity was seen in the rat cortex after brain injury site from 15 minutes to 72 hours after the trauma (19). Serum sFas, TNF- α levels can be useful as biochemical markers for early selection of patients at risk of deterioration after advanced degree of traumatic brain injury (20 and 21).

On conclusion, group of biochemical could be used in estimation of postmortem time interval for each time point.

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Review on Immune Tolerance Mechanism and Physiology

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Abstract- Immunological tolerance is classified into central tolerance or peripheral tolerance depending on where the state is originally induced in the thymus and bone marrow (central) or in other tissues and lymph nodes (peripheral). The mechanisms by which these forms of tolerance are established are distinct, but the resulting effect is similar. The recognition of antigens by the immature B cells in the bone marrow is critical to the development of immunological tolerance to self. This process produces a population of B cells that do not recognize self-antigens but may recognize antigens derived from pathogens or non self. T cells are selected for survival much more rigorously than B cells. They undergo both positive and negative selection to produce T cells that recognize self-MHC molecules but do not recognize self-peptides. Since, central tolerance is not 100% efficient, mechanisms of peripheral T-cell tolerance are required to prevent autoimmunity. Active peripheral tolerance is maintained by numerous types of regulatory T cells, the best known of which are FoxP3+ Tregs that develop naturally in the thymus or can be induced in the periphery. Central tolerance is the main way the immune system learns to discriminate self from non-self which is clearly stated in the case of early embryo communication within placental barrier. In did so with the natural balance rule the immune tolerance is play an important role on normal physiology to occur.

Keywords: autoimmunity, central, immune, peripheral, physiology, tolerance.

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Review on Immune Tolerance Mechanism and Physiology

Melese Yilma

Abstract- Immunological tolerance is classified into central tolerance or peripheral tolerance depending on where the state is originally induced in the thymus and bone marrow (central) or in other tissues and lymph nodes (peripheral). The mechanisms by which these forms of tolerance are established are distinct, but the resulting effect is similar. The recognition of antigens by the immature B cells in the bone marrow is critical to the development of immunological tolerance to self. This process produces a population of B cells that do not recognize self-antigens but may recognize antigens derived from pathogens or non self. T cells are selected for survival much more rigorously than B cells. They undergo both positive and negative selection to produce T cells that recognize self-MHC molecules but do not recognize self-peptides. Since, central tolerance is not 100% efficient, mechanisms of peripheral T-cell tolerance are required to prevent autoimmunity. Active peripheral tolerance is maintained by numerous types of regulatory T cells, the best known of which are FoxP3⁺ Tregs that develop naturally in the thymus or can be induced in the periphery. Central tolerance is the main way the immune system learns to discriminate self from non-self which is clearly stated in the case of early embryo communication within placental barrier. In did so with the natural balance rule the immune tolerance is play an important role on normal physiology to occur.

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I. INTRODUCTION

Immunological tolerance is describes a state of unresponsiveness of the immune system to substances or tissue that has the capacity to elicit an immune response (Michael and Ronald, 2011). Immune tolerance encompasses the range of physiological mechanisms by which the body reduces or eliminates an immune response to particular agents (Warrington *et al.*, 2011). It is used to describe the phenomenon underlying discrimination of self from non-self, suppressing allergic responses, allowing chronic infection instead of rejection and elimination, and preventing attack of fetuses by the maternal immune system (Tizared, 2013). Diverse innate immune cells including NK cells, DC and mast cells exert a variety of immune-regulatory mechanisms important for the induction of tolerance. These mechanisms include regulating T-cell activation and differentiation through cytokine production, the elimination of donor APCs, and

inhibition or killing of effector T cells (Murphy *et al.*, 2011).

Historically the phenomenon of immune tolerance was first described by Ray D. Owens in 1945, who noted that dizygotic twin cattle sharing a common placenta interestingly shared a stable mixture of each other's red blood cells and retained that mixture throughout life. Although Owens did not use the term immune tolerance, his study showed the body could be tolerant of these foreign tissues. This observation was experimentally validated by Rupert E. Billingham and Peter Medawar in 1953, which showed by injecting foreign cells into fetal or neonatal mice, they could become accepting of future grafts from the same foreign donor. Interestingly, though, they were not thinking of the immunological consequences of their work at the time. However, these discoveries and the host of allograft experiments and observations of twin chimerism they inspired were seminal for the theories of immune tolerance formulated by Sir Frank McFarlane Burnet and Frank Fenner, who was the first to propose the deletion of self-reactive lymphocytes to establish tolerance, now termed clonal deletion. Burnet and Medawar were ultimately credited for the discovery of "acquired immune tolerance" and shared the Nobel Prize in Physiology or Medicine in 1960 (Pelanda *et al.*, 1996; Hardy *et al.*, 1991).

Immune tolerance is important for normal physiology. Central tolerance is the main way the immune system learns to discriminate self from non-self. Peripheral tolerance is key to preventing over reactivity of the immune system to various environmental entities like allergens, gut microbes, and others (Tizared, 2013). Deficits in central or peripheral tolerance also cause autoimmune disease, resulting in syndromes such as systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes, autoimmune polyendocrine syndrome type 1 (APS-1), and immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), and potentially contribute to asthma, allergy, and inflammatory bowel disease (Hardy *et al.*, 1991). Immune tolerance, however, also has its negative tradeoffs. It allows for some pathogenic microbes to successfully infect a host and avoid elimination. In addition, inducing peripheral tolerance in the local microenvironment is a common survival strategy for a number of tumors that prevents their elimination by the host immune system (Roberta and Torres, 2015). Therefore the objective of this paper

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is to review you with the concept of immunological tolerance in physiology.

II. CENTRAL AND PERIPHERAL TOLERANCE

Immunological tolerance is classified into central tolerance or peripheral tolerance depending on where the state is originally induced in the thymus and bone marrow (central) or in other tissues and lymph nodes (peripheral) (Roberta and Torres, 2015; Tizared, 2013). The mechanisms by which these forms of tolerance are established are distinct, but the resulting effect is similar.

Central tolerance refers to the tolerance established by deleting autoreactive lymphocyte clones before they develop into fully immunocompetent cells (Tizared, 2013). It occurs during lymphocyte development in the thymus and bone marrow for T and B lymphocytes, respectively. In these tissues, maturing lymphocytes are exposed to self-antigens presented by medullary thymic epithelial cells, thymic dendritic cells, or bone marrow cells (Warrington *et al.*, 2011). At the level of bone marrow the germ line gene rearrangement and inability call to express surface marker (B cell, 220B/CD44) is order for progenitor B cell tolerance in the bone marrow (Roberta and Torres, 2015).

Those lymphocytes that have receptors that bind strongly to, or "recognize," self-antigens are removed by induction of apoptosis of the autoreactive cells, or by induction of anergy, a state of non-activity. Weakly autoreactive B cells may also remain in a state of immunological ignorance where they simply do not respond to stimulation of their B cell receptor. Some weakly self-recognizing T cells are alternatively differentiated into natural regulatory T cells (nTreg cells), which act as sentinels in the periphery to calm down potential instances of T cell autoreactivity. This process of negative selection ensures that T and B cells that could initiate a potent immune response to the host's own tissues are eliminated while preserving the ability to recognize foreign antigens (Roberta and Torres, 2015).

Peripheral tolerance develops after T and B cells mature and enter the peripheral tissues and lymph nodes. It is established by a number of partly overlapping mechanisms that mostly involve control at the level of T cells, especially CD4⁺ helper T cells, which orchestrate immune responses and give B cells the confirmatory signals they need in order to produce antibodies (Nemazee, 2017). Inappropriate reactivity toward normal self-antigen that was not eliminated in the thymus can occur, since the T cells that leave the thymus are relatively but not completely safe (Warrington *et al.*, 2011). Some will have receptors (TCRs) that can respond to self-antigens that are present in such high concentration outside the thymus that they can bind to "weak" receptors and the T cell did not encounter in the thymus (such as, tissue-specific

molecules like those in the islets of Langerhans, brain, or spinal cord not expressed by AIRE in thymic tissues). Those self-reactive T cells that escape intrathymic negative selection in the thymus can inflict cell injury unless they are deleted or effectively muzzled in the peripheral tissue chiefly by nTreg cells (Tizared, 2013).

a) *Mechanisms of B Cell Tolerance*

The recognition of antigens by the immature B cells in the bone marrow is critical to the development of immunological tolerance to self. This process produces a population of B cells that do not recognize self-antigens but may recognize antigens derived from pathogens (non-self). Immature B cells expressing only surface Ig^M molecules undergo negative selection by recognizing self-molecules present in the bone marrow. This antigen induced loss of cells from the B cell repertoire is known as clonal deletion (Akiyama *et al.*, 2005).

B cells may encounter two types of antigen, multivalent cell surface antigens or low valence soluble antigens: (1) When immature B cells express surface Ig^M that recognizes ubiquitous self - cell-surface (i.e. multivalent) antigens, such as those of the MHC they are eliminated by a process known as clonal deletion. These B cells are believed to undergo programmed cell death or apoptosis. (2) Immature B cells that bind soluble self-antigens (i.e. low valence) do not die but their ability to express Ig^M on their surfaces is lost (as a result of the downregulation in receptor synthesis due to the development of receptor tolerance similar to the process seen in drug tolerance through constant exposure to self-antigen). Thus, they migrate to the periphery only expressing IgD (pushed by the division of additional B cells) and are unable to respond to antigen. These B cells are said to be anergic (Nemazee, 2017; Tizared, 2013; Akiyama *et al.*, 2005; Klinman, 1996).

Only B cells that do not encounter antigen whilst they are maturing in the bone marrow can be activated after they enter the periphery. These cells bear both IgM and IgD receptors and constitute the repertoire of B cells that recognize foreign antigen. Even if mature self-reacting B cells were to survive intact, they would very rarely be activated. This is because B cells need co-stimulatory signals from T cells as well as the presence of its recognized antigen to proliferate and produce antibodies (Peripheral tolerance). If mature peripheral B cells encounter multivalent antigen (e.g. cell surfaces) they are eliminated via apoptosis. If mature B cells recognize soluble antigen in the periphery in the absence of T cell help, they lose surface IgM receptors and become anergic (Macauley *et al.*, 2013; Tizared, 2013).

b) *Mechanisms of T cell tolerance*

T cells are selected for survival much more rigorously than B cells. They undergo both positive and

negative selection to produce T cells that recognize self-major histocompatibility complex (MHC) molecules but do not recognize self-peptides. T cell tolerance is induced in the thymus (Tizared, 2013). Positive selection occurs in the thymic cortex. This process is primarily mediated by thymic epithelial cells, which are rich in surface MHC molecules. If a maturing T cell is able to bind to a surface MHC molecule in the thymus, it is saved from programmed cell death; those cells failing to recognize MHC on thymic epithelial cells will die. Thus, positive selection ensures that T cells only recognize antigen in association with MHC. This is important because one of the primary functions of T cells is to identify and respond to infected host cells as opposed to extracellular pathogens (Bose *et al.*, 2003). The process of positive selection also determines whether a T cell ultimately becomes a CD4+ cell or a CD8+ cell: prior to positive selection, all thymocytes are doubling positive (CD4+CD8+) i.e. bear both co-receptors. During positive selection they are transformed into CD4+CD8- or CD8+CD4- T cells depending on whether they recognize MHC II or MHC I, respectively (Bose *et al.*, 2003).

T cells also undergo negative selection in a process analogous to the induction of self-tolerance in B cells, this occurs in the cortex, at the cortico-medullary junction, and the medulla (mediated in the medulla predominately by medullary thymic epithelial cells (mTECs) and dendritic cells). mTEC display "self" antigens to developing T-cells and signal those "self-reactive" T-cells to die via programmed cell death (apoptosis) and thereby deleted from the T cell repertoire. This process is highly dependent on the ectopic expression of tissue specific antigens (TSAs) which is regulated by the autoimmune regulator (Lahl *et al.*, 2007).

This clonal deletion of T cells in the thymus cannot eliminate every potentially self-reactive T cell; T cells that recognize proteins only found at other sites in the body or only at certain times of development (e.g. after puberty) must be inactivated in the periphery. In addition, many self reactive T cells may not have sufficient affinity (binding strength) for the self antigen to be deleted in the thymus (Tizared, 2013; Lahl *et al.*, 2007).

The first opportunity to eliminate self-reactive T cells occurs in the thymus, where thymocytes undergoing high avidity interactions with APCs presenting a self-antigen are eliminated by clonal deletion. Thymocytes experiencing interactions with APCs that while not strong enough to trigger cell death are sufficiently strong to indicate an unacceptable level of self-reactivity may undergo induction of anergy, TCR revision, or be diverted into alternative lineages, such as FoxP3+ regulatory T cells or CD8α T cells instead of undergoing deletion (Akirav *et al.*, 2011).

Despite the numerous mechanisms of central tolerance, it is clear that self-reactive T cells still escape to the periphery. This may occur stochastically for some thymocytes simply because they did not encounter the limited number of APCs expressing their cognate ligand as they matured in the thymus. In other cases, the avidity between the self-reactive thymocyte and the APC presenting the self-antigen may not be quite high enough to trigger the normal mechanisms of central tolerance. Low avidity interactions may occur because the TCR has relatively low affinity for the peptide/MHC complex. Alternatively, the peptide may have low affinity for the MHC molecule, and this unstable interaction may result in a low abundance of peptide/MHC complexes available on the APC cell surface (Kretschmer *et al.*, 2005).

Normally, these low avidity interactions would not pose a threat when the T cells enter the periphery, as the threshold for T cell activation in the periphery is believed to be higher than the threshold for induction of central tolerance. However, T cells with low avidity for their self-antigen/ MHC complex could contribute to autoimmunity if the abundance of self-antigen/ MHC complexes increases in the periphery relative to the amount found in the thymus, or a post-translational modification of the self-antigen occurs in the periphery that increases the affinity of the TCR for the self-antigen/MHC complex (Gardner *et al.*, 2009).

Furthermore, antigens that are developmentally expressed may not be present in the thymus when some thymocytes are subjected to negative selection, resulting in a failure to induce central tolerance. Cells expressing these antigen/MHC complexes later in life could become the targets of self-reactive T cells that matured in the thymus and entered peripheral circulation prior to the expression of the self-antigen (Shimon *et al.*, 2008).

c) *Immune Tolerance in Physiology the Case of Maternal Embryo Recognition*

Immune tolerance is important for normal physiology. Central tolerance is the main way the immune system learns to discriminate self from non-self (Roberta and Torres, 2015). The fetus has a different genetic makeup than the mother, as it also translates its father's genes, and is thus perceived as foreign by the maternal immune system. Women who have borne multiple children by the same father typically have antibodies against the father's red blood cell and major histocompatibility complex (MHC) proteins. However, the fetus usually is not rejected by the mother, making it essentially a physiologically tolerated allograft (Noronha and Actczak, 2010).

It is thought that the placental tissues which interface with maternal tissues not only try to escape immunological recognition by downregulating identifying MHC proteins but also actively induce a marked

peripheral tolerance. Placental trophoblast cells express a unique Human Leukocyte Antigen (HLA-G) that inhibits attack by maternal NK cells. These cells also express IDO, which represses maternal T cell responses by amino acid starvation (Petroff *et al.*, 2002).

Maternal T cells specific for paternal antigens are also suppressed by tolerogenic DCs and activated iTregs or cross-reacting nTregs. Some maternal Treg cells also release soluble fibrinogen-like proteins 2 (sFGL₂), which suppresses the function of DCs and macrophages involved in inflammation and antigen presentation to reactive T cells. These mechanisms altogether establish an immune-privileged state in the placenta that protects the fetus. A break in this peripheral tolerance results in miscarriage and fetal loss (Hunt and Robertson, 1996).

The maternal immune system has to balance the opposing needs of maintaining robust immune reactivity to protect both mother and fetus from invading pathogens, while at the same time tolerating highly immunogenic paternal alloantigens in order to sustain fetal integrity. Regulatory T cells are responsible for the establishment of tolerance by modulating the immune response and uterine natural killer cells direct placentation by controlling trophoblast invasion. A variety of other cell types, including decidual stromal cells, dendritic cells, and immunomodulatory multipotent mesenchymal stromal cells, are found at the fetal-maternal interface. These cells conspire to establish a suitable environment for fetal development without compromising systemic immunity (Suano *et al.*, 2011). Defects in any of these components can lead to gestational failure despite successful fertilization.

III. CONCLUSIONS

Immunological tolerance is classified into central tolerance or peripheral tolerance depending on where the state is originally induced in the thymus and bone marrow (central) or in other tissues and lymph nodes (peripheral). The mechanisms by which these forms of tolerance are established are distinct, but the resulting effect is similar. The recognition of antigens by the immature B cells in the bone marrow is critical to the development of immunological tolerance to self. This process produces a population of B cells that do not recognize self-antigens but may recognize antigens derived from pathogens (non-self). T cells are selected for survival much more rigorously than B cells. They undergo both positive and negative selection to produce T cells that recognize self-MHC molecules but do not recognize self-peptides. Because central tolerance is not 100% efficient, mechanisms of peripheral T-cell tolerance are required to prevent autoimmunity. Active peripheral tolerance is maintained by numerous types of regulatory T cells, the best known of which are FoxP3⁺ Tregs that develop naturally in the thymus or can be

induced in the periphery. Immune tolerance is important for normal physiology. Central tolerance is the main way the immune system learns to discriminate self from non-self which is clearly stated in the case of early embryo communication within placental barrier. Recent research reveals the cellular and molecular basis of immune tolerance development and function and implicates deregulations of immunological disease and treatment option further study and recommendation is must to have comprehensive immunological idea on normal physiology and autoimmune disorder treatment.

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Stop the Time for Your Skin: A Search for Aging Process

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Keywords: *aging, anti-aging, skin elasticity, herbal medicine, testosterone, estrogen, physical characteristic, hyaluronic acid, photo aging.*

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Stop the Time for Your Skin: A Search for Aging Process

Harun Cerit ^α, Engin Kozak ^α, Ipek Buyukipekci ^ρ & Ayşe Zehra Aroguz ^ω

Abstract- One of the most common yet mis and poorly understood topic is the ageing process and how it affects the body. Aging is a complex phenomenon that emerges in the coming years. Skin aging can be classified as intrinsic aging and extrinsic aging (photoaging). The skin changes over time due to a decrease in hormone levels, chronic sun exposure, chronic inflammation and many other reasons. It occurs at the microscopic level with physiological, histological and metabolic changes, at macroscopic level with wrinkles, dryness, loss of elasticity and stain formation. Delaying skin aging and alleviating the signs of aging with cosmetic products is one of the most important tasks of cosmetic science. The use of topically applied cosmeceuticals containing ingredients that affect deep biological functions has increased significantly in recent years. Anti aging cosmetic products used in facial care should be scientifically proven products whose efficacy and reliability are intended to show anti-aging effects using different biologically active ingredients. It is the intention of this article to review which biomolecules cause aging, why they do it, and which herbs can prevent unwanted problems caused by aging.

Keywords: aging, anti-aging, skin elasticity, herbal medicine, testosterone, estrogen, physical characteristic, hyaluronic acid, photo aging.

I. WHAT IS AGING PROCESS?

Aging is the continual processes of wear and tear in body which affect us both physically and mentally. Aging is an inevitable life fact and one of the most reputable problem of the current century. With its symptoms, almost all people have a fear of becoming old. Although symptoms of aging change person to person, loss of beauty in aesthetical features appears in general. While getting old, people face with growing white hair and hair loss, vision loss, hearing loss, skin deterioration, decay in posture, etc. In addition to these symptoms of physical appearance, the ones that cause health problems are far more important. While people get old, they become more susceptible to the age-related diseases like cancer, diabetes, cardiac dysfunctions, Alzheimer and many more. Those reasons can result in the decrease of self-confidence and life quality. Because of that, being young is the best way to live a healthy life with a sharp memory, strong muscles, good-looking physical appearance, good health, healthy brain and efficient immune system.

Even if people argue over what drives any or all of those processes, they all seem to agree on how they

affect us; they make us prone to falling apart. Not surprisingly, aging has a long history of humankind and their cultural traditions.^[1]

II. THEORIES THAT EXPLAINS AGING PROCESS

Even though there are a lot of theories for the explanation of the process of aging, none of them are fully convincing. Traditional ones support that aging is not an adaptation or genetically programmed. Modern biological theories of aging divided into two main categories which are programmed and damage or error. Programmed theory divided into three sub-categories but the main idea is aging is a continuation of growth and development which follows a biological timetable. In damage and error theories, aging is a result of an environmental assault which induces cumulative damage on living organisms.^[2]

The free radical theory proposes that tissues and cells are damaged by the oxidative respiration as a result of aerobic metabolism. There are couple of evidence to support that: lifetime of the species depends on the metabolism rate and antioxidant activity which is protective to the body; increased production and expression of antioxidative enzymes can affect the lifetime of species; cellular level of free radical damage that is caused by some of the molecules, increases in time; additional or excessive calorie intake which causes extra free radicals also reduce the lifetime.^[3]

III. MAIN BIOCHEMICAL PROCESSES IN ANTIAGING

The biochemical processes that involved in aging are oxidation, glycation, and methylation. Other appropriate processes are chronic inflammation and hormonal deregulation.

a) Oxidation

Free radicals are produced by oxidation in oxygen metabolism within cells. They are a simple compound with an electron missing from their chemical structure which makes them unstable. Because of that, they seek out other chemical structures, so that they can acquire an electron and make the other structure unstable. The defense mechanism against free radicals is inactivating them after the production, removing them from antioxidants and increasing the elimination of

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material which already damaged by free radicals. Free radicals damage the cell membrane which is composed of lipids and proteins. Their interaction with proteins and lipids causes the production of malondialdehyde which is very harmful, contributing to another aging process called glycation.

b) *Glycation and Carnosine*

Glycation is the attachment of glucose, fructose, and other sugar molecules to proteins. The binding between sugar and protein results in a cross-linking of proteins. Cross-linked proteins cause more damage by reacting with free radicals and other toxins. In addition to proteins, glycation reactions affect DNA too, resulting in cross-linked DNA molecules which are no use at all.

Dipeptide carnosine (β-alanyl-L-histidine), discovered a century earlier, is found in animal tissue, especially in muscle and brain, and sometimes in millimolar concentrations. There is increasing evidence that carnosine may be an effective anti-gelling agent, at least in model systems and cultured cells. Dipeptide can be used to inhibit the formation of protein carbonyls and to inhibit the formation of crosslinks induced by reducing sugars and other reactive aldehydes, malondialdehyde, and methylglyoxal.^[4]

Carnosine has some healing effects on aging, cellular and all animal levels. Carnosine suppresses aging in cultured human fibroblasts and even plays older cells. More recently, carnosine has been shown to protect the telomeres of cultured cells against oxidative damage. The beneficial effects of carnosine aging-accelerated mice on the survival, *Drosophila* and rodent fibroblasts was also described.^[4]

c) *Methylation*

Methylation is the addition of methyl groups to proteins, DNA, and other molecules to keep them in good and active condition. Increased level of homocysteine reflects low methylation that is found in diabetes and heart disease. Increased intake of methylators reduces the risk of these diseases.

d) *Chronic Inflammation*

Chronic inflammation is the process of destruction of body tissues by toxic chemicals. Results in dementia, thickening of the arteries, diabetes, hormonal imbalance and others.

Recent data indicate that systemic aging can be affected by inflammation with neuroendocrine markers³. At a tissue level, inflammation may contribute to senescence^{5,6,7} in progeria mouse models⁴ and DNA damage, indicating synergistic interactions between DNA damage responses (DDRs) and inflammatory signals.

Latest data show that systemic aging might be influenced by inflammation via neuro-endocrine signaling^[5]. On a tissue level, inflammation might contribute to aging in mouse skin^[6] and in DNA

damage-driven progeria mouse models^[7,8,9], suggesting synergistic interactions between DNA damage responses (DDRs) and inflammatory signals.

e) *Hormonal Deregulation*

Hormonal deregulation is an important factor in aging since hormones are needed to be replaced and reactivated during aging to prevent the body from falling apart^[10].

f) *Proteins*

The most common symptom of aging at the molecular level is the accumulation of altered proteins both in and out of cells; The modifications may be in either the polypeptide chain length or the amino acid composition. Modified proteins are produced continuously, both biosynthetically and synthetically.

Altered proteins are also associated with many age-related pathologies such as Alzheimer's disease (AD), Parkinson's disease (PD), cataractogenesis, atherosclerosis, diabetic secondary complications, etc. Consequently, it is thought that these accumulations are possibly causative to much age-related pathology and interventions to either prevent the production of the altered protein forms or facilitate their removal could delay certain age-related diseases^[4].

g) *Photoaging*

Chronic exposure to the sun leads to the photoaging of human skin, a process characterized by clinical, histological, and biochemical changes that are chronologically elongated. In recent years significant progress has been made in resolving the underlying mechanisms of photodamage. Recently, the induction of matrix metalloproteinases as a consequence of activator protein (AP)-1 and nuclear factor (NF)-κB activation and mitochondrial DNA mutations has been described^[11].

IV. WHAT IS ANTI-AGING?

Anti-aging means prevention or limitation of the process of becoming old. Furthermore, the meaning of anti-aging changes in medical and scientific fields. In the scientific field, anti-aging refers to prevention, deceleration, or reversion of aging process. In the medical field, it refers to early detection, prevention, and treatment of age-related diseases. For instance, heart diseases are age related and a treatment method that do not effect on the aging process can help people to live longer and healthier lives. The treatment method is anti-aging according to medical fields. However, scientific fields do not approve^[12].

a) *Applications of anti-aging*

There are a lot of different types of applications of anti-aging like;

- Without addressing aging, treatment a specific disease.

- Slowing down aging process by preventing or delaying physiological decline and regaining lost functional abilities.
- Cosmetic treatments.
- Altering our bodies to improve the basic molecular and genetic processes of maintenance and repair to make them work more efficient, and longer.
- Use of the body's intrinsic capacity for self-maintenance and repair^[13].

V. WHAT ARE THE KEY MOLECULES THAT USED IN ANTI-AGING?

• Estrogen

Sex hormones are involved in changes in skin texture, elasticity, and growth of hair. The effects of estrogen on human skin studied and results shows that it has a delaying or preventing effect on skin aging^[14].

In the skin, estrogens affect thickness, wrinkle formation and moisture. Estrogens may enhance glycosaminoglycan (GAG), such as Hyaluronic Acid, to maintain fluid balance and structural integrity. At the same time, they can increase the production of collagen, maintaining epidermal thickness and allowing the skin to remain fuller, hydrated and wrinkle-free. It is not the only external feature that utilizes skin from estrogens. In addition to revealing a fuller, healthy skin, estrogens can make your hair longer and healthier^[15].

a) Estrogens and the skin

Loss of estrogen in the menopause has a profound effect on the skin. Postmenopausal women have shown that estrogen treatment increases collagen content, skin thickness and elasticity, and the effect of estrogen on skin water content is also promising^[16].

• Testosterone

Some of the effects of testosterone treatments are studied and by the results, it is clear that testosterone treatment to increase lean body mass, decrease visceral fat mass, increase bone mineral density, and decrease total cholesterol for aging hypogonadal men^[17]. Rougher hair, thicker and oilier skins, and usually a sign of skin aging, is linked to testosterone. Female paternal alopecia or baldness is associated with increased androgen levels and is the most common cause of hair loss in women. With age, the ratio of estrogen-androgen 3 becomes unbalanced, and changes occur after menopause. Since androgens and especially testosterone are involved in skin sebum production, increased fatness or even adult acne may occur when hormones become unbalanced during menstruation or menopause in women. The effects of androgens on the skin are important in both male and female patients, both of which may be affected by varying levels of androgens^[15].

b) Testosterone delays vascular smooth muscle cell (VSMC) senescence and inhibits collagen synthesis

In this study, muscular mechanism of testosterone in protecting against VSMC senescence was found. Results showed that testosterone plays a fundamental role in cell senescence which could be a critical mechanism for delaying vascular aging^[18]

c) Testosterone increases renal anti-aging klotho gene expression via the androgen receptor-mediated pathway

They found that renal klotho mRNA and protein expression were significantly reduced in the aging kidneys of male mice that have a lower serum testosterone level and lower AR expression than young mice. Moreover, testosterone supplement up-regulated both klotho and AR expression in testosterone-deficient ORX mice and in NRK52E cells, suggesting that renal klotho mRNA expression may be regulated by testosterone in an AR-dependent mechanism^[19]

• Phytochemicals

Phytochemicals are secondary plant metabolites that present in food, fruits, vegetables, nuts, cocoa, beverages and many more. Abilities of many phytochemicals have been studied and they have been found that they have an effect on the prevention and treatment of diseases like cancer, cardiovascular disease, diabetes, obesity, Alzheimer and other neurological dysfunctions^[20]

Betanin is a sub group of one of the seven main categories of phytochemicals, betalains. It presents in beets and chard.

d) Effect of betanin on a rat paraquat-induced acute lung injury (ALI)

In this study, paraquat was injected intraperitoneally at a single dose and betanin was orally administered 3 days before and 2 days after paraquat administration. Rats were sacrificed 24 hours after the last betanin dosage, and lung tissue and bronchoalveolar lavage fluid (BALF) were collected. In rats treated only with paraquat, extensive lung injury characteristic of ALI was observed. In rats treated with betanin, paraquat-induced ALI was attenuated in a dose-dependent manner^[21]

e) Effect of betanin on reducing accumulation and crosslinking of collagen in high-fructose-fed rat heart

Because of its antagonizing effect with insulin resistance, according to this study; betanin treatment reduces the cardiac collagen accumulating, cross-linking and inhibited profibrotic factor-TGF-B1 and CTGF protein expression in fructose-fed rat heart^[22]

f) Betanin attenuates carbon tetrachloride (CCl₄)-induced liver injury in common carp (*Cyprinus carpio* L.)

This study, protective effect of betanin against liver injury induced by carbon tetrachloride (CCl₄) in

common carp was investigated. The fish were treated with betanin in fodder throughout the experiment. After 20 days of treatment, the fish were intraperitoneally injected with CCl₄, and were killed three days after CCl₄ intoxication, and then, histological and biochemical assays were performed. Results showed that CCl₄-induced liver CYP2E1 activity, oxidative stress, and injury, as indicated by the depleted glycogen storage, increased serum aspartate aminotransferase (AST)/alanine aminotransferase (ALT) activities and liver histological damage. Compared with the CCl₄ control group, the betanin-treated groups exhibited, increased liver antioxidative capacity (increased glutathione level and superoxide dismutase and catalase activities), increased liver glycogen storage, reduced CYP2E1 activity, decreased malondialdehyde level, and reduced serum AST/ALT activities, with significant differences in the 2 and 4 % groups ($p < 0.05$). In conclusion, betanin attenuates CCl₄-induced liver damage in common carp. Moreover, the inhibition of CYP2E1 activity and oxidative stress may have significant roles in the protective effect of betanin^[23]

- *Hyaluronic acid*

Hyaluronic acid is a polysaccharide which is a part of the glycosaminoglycan family. It consists of a basic unit of two sugars, glucuronic acid, and N-acetylglucosamine. As a result of senescence, size of the HA polymers in the skin decreases over time^[24]. Thus the epidermis loses the principle molecule responsible for binding and retaining water molecules, resulting in loss of skin moisture.

- g) *Hyaluronic acid and wound healing*

In this study, an experiment model of ethanol-induced dermatotoxicity and hepatotoxicity using normal human keratinocytes and normal human hepatocytes that preserve inducible cytochrome p450 activities was developed. Results showed that hyaluronic acid could be used safely for skin regeneration. Both alone and combination with herbals choices are viable^[25,26].

- *Melatonin*

Melatonin has a favorable influence on the aging process because it has an inverse effect with regard to body weight; food restriction raises the levels of melatonin and decreases its age-related decrease. With increasing age comes a decrease of melatonin production, which may have a connection to sleep disorders suffered by elderly people. It also was shown that melatonin can prevent the tumor development and growth. Interestingly, a study showed that patients with the tumor has a low level of melatonin compared to healthy individuals^[27,28].

- h) *What are the herbs that can or might affect aging process*

Some of the herbs defining as anti-aging herbs. Especially in Asian countries, medical herbs have a long history of usage as medicine. Anti-aging herbs have different effects on the body; they can boost the level of vital energy, act as food to provide essential nutrients, can be used for therapeutic effects^[29].

Aloe vera L. (Family: Liliaceae) Aloeverais known for its medicinal uses for a very longtime. The usefulness of the plant was proven for minor burns and sunburns. However, medical and cosmetic effectiveness is still studying among scientist.

It has been shown that Alloin (1) and B inhibit *Clostridium histolyticum* collagenase in a reversible and reciprocal manner. Both aloe gel and alloin are also effective inhibitors of stimulated granulocyte MMPs^[30]. It has been reported to modulate melanogenesis through the competitive inhibition of aloesin [2-acetyl-8-beta-glucopyranosyl-7-hydroxy-5-methylchromone], tyrosinase, isolated from *A. vera*. Tyrosine hydroxylase and 3,4-dihydroxyphenylalanine oxidase activities of tyrosinase from normal human melanocyte cell lysates were inhibited by aloesin in a dose-dependent manner^[31].

Camellia japonica L. (Family: Theaceae) The anti-aging properties of *C. japonica* oil have been reported in human dermal fibroblast cells due to concentration in the analysis of the human COL1A2 promoter luciferase^[32]. Human type I procollagen synthesis was found to be induced by *C. japonica* oil while MMP-1 activity was inhibited. *C. japonica* oil can also retain trans-epidermal water loss (TEWL) without any side effects.

Camellia sinensis L. (Family: Theaceae) Originally cultivated in East Asia, this plant grows as large as a shrub. Sunscreen formulated with 2–5% green tea extract has been reported to protect UV irradiation induced photoaging, photoimmunosuppression, cutaneous erythema, thickening of the epidermis, over expression of CK5/6, CK16, MMP-2, MMP-9, etc.^[33]. A double-blind, placebo-controlled trial has been executed with moderate photoaging treated with either a combination regimen of 10% green tea cream and 300 mg twice-daily green tea oral supplementation or a placebo regimen for eight weeks to monitor the clinical and histologic appearance of photoaging skin^[34]. Patients treated with topical as well as oral combination regimens have shown histological improvement in tissue elasticity, but no clinically significant changes have been found and may require a longer reinforcement for clinically observable developments. Green tea polyphenols have been reported to be suitable sunscreen to protect the skin from adverse effects such as catechin, epigallocatechin, epigallocatechin-3-gallate, etc., caused by UV radiation, inflammation, oxidative stress and DNA damage. skin cancer risk^[35].

Curcuma longa L. (Family: Zingiberaceae) *C. longa* extract has been found to make potential changes in skin thickness, elasticity, pigmentation and wrinkles caused by long-term, low-dose UV-B irradiation in melanin-containing hairless mice^[36]. It prevents wrinkle and melanin formation and increases in the diameter and length of the skin blood vessels and reduces matrix metalloproteinase-2 (MMP-2) expression. For this reason, skin rash can be reduced with curcumin.

Glycine max L.Merr (Family: Fabaceae) Anthocyanin isolated from black soybean seed responsible for down-regulation of in vitro and in vivo UVB induced reactive oxygen species levels and apoptotic cell death through the prevention of caspase-3 pathway activation and reduction of proapoptotic Bax protein levels^[37]. Finding highlights that anthocyanin from the seed coat of black soybean is useful compounds to modulate UVB-induced photoaging.

Prunus dulcis Mill. (Family: Rosaceae) The role of almond oil in reducing the degradative changes induced in skin upon exposure to UV radiation has been proposed and illustrated that biochemical parameters, glutathione, and lipid peroxidation have been ameliorated by almond oil^[38].

Vaccinium uliginosum L. (Family: Ericaceae) Fruits of bog blueberry (*V. uliginosum*) are rich in anthocyanins like cyanidin-3-glucoside, petunidin-3-glucoside, malvidin-3-glucoside, and delphinidin-3-glucoside which have been documented for pigmentation and attenuation of photoaging through removal of reactive oxygen species (ROS) production and the resultant DNA damage responsible for activation of p53 and Bad in UV-B-irradiated human dermal fibroblasts^[39].

Zingiber officinale L. (Family: Zingiberaceae) Topical application of *Z. officinale* extract to hairless mouse skin significantly inhibited the wrinkle formation induced by chronic UV-B irradiation at a suberythemal dose accompanied by significant prevention of the decrease in the skin elasticity^[40].

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Epidemiological Study of African Horse Sickness in Sudan

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Abstract- A cross-sectional study was conducted from September 2016 to October 2017 to determine the seroprevalence and to investigate the risk factors for African horse sickness (AHS) in Sudan. A total of 920 equines (590 horses and 330 donkeys) were randomly selected and sampled. Competitive Enzyme Linked Immuno-Sorbent Assay (c-ELISA) was employed to detect antibodies to AHS virus. The overall seroprevalence was 72.2 %, while it was 80% in horses and 58.2% in donkeys. The univariate analysis of associations of potential risk factors with seroprevalence of AHS showed statistically significant ($p \leq 0.05$) results with state ($\chi^2 = 47.434$, $p < 0.001$), species ($\chi^2 = 50.163$, $p < 0.001$), sex ($\chi^2 = 26.206$, $p < 0.001$), housing ($\chi^2 = 26.477$, $p < 0.001$), vaccination ($\chi^2 = 44.466$, $p < 0.001$), breed ($\chi^2 = 57.256$, $p < 0.001$), water bodies ($\chi^2 = 26.271$, $p < 0.001$), Culicoides ($\chi^2 = 42.658$, $p < 0.001$), ticks ($\chi^2 = 23.608$, $p < 0.001$), activity of animal ($\chi^2 = 41.435$, $p < 0.001$), awareness of owner ($\chi^2 = 25.639$, $p < 0.001$), age ($\chi^2 = 20.186$, $p < 0.001$), health score ($\chi^2 = 12.038$, $p < 0.001$), pregnancy ($\chi^2 = 3.249$, $p = 0.0355$), and infection with other disease ($\chi^2 = 14.637$, $p < 0.001$). However, the risk factors of presence of other animals and previous infection with AHS did not show statistically significant ($p > 0.05$) associations.

Keywords: african horse sickness, AHS, c-ELISA, seroprevalence, risk factors, equines, horses, donkeys, sudan.

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Epidemiological Study of African Horse Sickness in Sudan

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Abstract- A cross-sectional study was conducted from September 2016 to October 2017 to determine the seroprevalence and to investigate the risk factors for African horse sickness (AHS) in Sudan. A total of 920 equines (590 horses and 330 donkeys) were randomly selected and sampled. Competitive Enzyme Linked Immuno-Sorbent Assay (c-ELISA) was employed to detect antibodies to AHS virus. The overall seroprevalence was 72.2 %, while it was 80% in horses and 58.2% in donkeys. The univariate analysis of associations of potential risk factors with seroprevalence of AHS showed statistically significant ($p \leq 0.05$) results with state ($\chi^2 = 47.434$, $p < 0.001$), species ($\chi^2 = 50.163$, $p < 0.001$), sex ($\chi^2 = 26.206$, $p < 0.001$), housing ($\chi^2 = 26.477$, $p < 0.001$), vaccination ($\chi^2 = 44.466$, $p < 0.001$), breed ($\chi^2 = 57.256$, $p < 0.001$), water bodies ($\chi^2 = 26.271$, $p < 0.001$), *Culicoides* ($\chi^2 = 42.658$, $p < 0.001$), ticks ($\chi^2 = 23.608$, $p < 0.001$), activity of animal ($\chi^2 = 41.435$, $p < 0.001$), awareness of owner ($\chi^2 = 25.639$, $p < 0.001$), age ($\chi^2 = 20.186$, $p < 0.001$), health score ($\chi^2 = 12.038$, $p < 0.001$), pregnancy ($\chi^2 = 3.249$, $p = 0.0355$), and infection with other disease ($\chi^2 = 14.637$, $p < 0.001$). However, the risk factors of presence of other animals and previous infection with AHS did not show statistically significant ($p > 0.05$) associations. Furthermore, in the multivariate analysis only state (OR = 4.909, $p = 0.017$), breed (OR = 2.532, $p = 0.004$), species (OR = 3.776, $p = 0.017$), water bodies (OR = 2.172, $p = 0.033$), and vaccination (OR = 17.298, $p < 0.001$) were found to be statistically significantly ($p \leq 0.05$) associated with seroprevalence of AHS.

Keywords: african horse sickness, AHS, c-ELISA, seroprevalence, risk factors, equines, horses, donkeys, sudan.

1. INTRODUCTION

African Horse Sickness (AHS) is a vector-borne viral disease of horses, mules, and donkeys. The clinical signs and lesions occur as a result of

increased vascular permeability and are characterized by an impairment of the respiratory and circulatory system (Radostits et al., 2007). AHS was first recognized in southern Africa, with the first outbreak recorded in 1719, when more than 1700 animals died. Although endemic in equines in Sub-Saharan Africa, outbreaks have also been recorded in North Africa, the Middle and Near East and southern Europe (MacLachlan and Guthrie 2010). AHS virus is transmitted by the biting midges *Culicoides*. *Culicoides simcolais* is the field vector of AHS virus although *Culicoides bolitinos* has been shown to play an important role in the transmission of AHS virus in the cooler upper highlands of South Africa (Meiswinkel and Paweska 2003). The highest incidence of the disease usually occurs in the late summer and early autumn in years when the climatic conditions favor an abundance of *Culicoides* midges (Coetzer and Guthrie 2004).

Clinically, the disease is characterized by an acute pulmonary form, a cardiac form or sub-acute form, mixed form and a mild form known as horse sickness fever (Upadhyaya, 2011). AHS is one of the viral diseases characterized by up to 95, 50 and 10% mortality rates in horses, mules, and donkeys, respectively (OIE, 2008).

In a study performed in horses and donkeys from southern Darfur and Khartoum states, the sera were analyzed by passive haemagglutination test and serum neutralization test, the overall seroprevalence was 42.64% and 27.75%, respectively (Ihsan 2004). In different regions of Ethiopia, Tesfaye et al. (2012), Kassa (2006), Demissie (2013), Ende et al. (2013), Molalegne et al. (2010), and Yeshitila and Bekele (2017) found that the apparent seroprevalence of AHS was 24.60%, 23%, 33.04%, 46.2%, 25% and 23.47%, respectively. There were no significant variations ($P > 0.05$) among age groups and sex for seroprevalence of the disease (Ende et al. 2013, Kassa 2006, Tesfaye et al. 2012). In the Khartoum state of Sudan, Abu Elzein et al. (1989) found that the seroprevalence in donkeys was 98% for AHS by using the micro AGID test. Also, in the same area antibodies to AHS virus were detected in horses, donkeys, goats, cattle and Dorcas gazelle in a rate of 78.9%, 76.7%, 20%, 15% and 11.1%, respectively (Elghazali and Ali 2013). In Zimbabwe, results indicated a higher seroprevalence of the disease in the late rainy season (68.9%) compared to other seasons. Age and

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vaccination of horses were found to be significant factors (Gordon et al., 2013).

Sudan livestock population for the year 2009 was 41.65 million cattle, 51.55 million sheep, 43.27 million goats, 4.52 million camels, 7.51 million donkeys and 784 thousand horses (Statistical Bulletin for Animal Resources 2009). There were previous studies conducted to investigate AHS in Sudan (Eisa 1974; Abu Elzein et al. 1989; Ihsan 2004; Elghazali and Ali 2013). However, these studies were conducted in few states, and small sample sizes were investigated. Hence, there was a gap of research information and published studies which point out the seroprevalence of AHS and investigate its association with potential risk factors. Therefore, this study was conducted to fill the information gap regarding the seroprevalence and potential risk factors associated with the disease in Sudan.

II. MATERIALS AND METHODS

a) Study design

The study was designed as a cross-sectional observational study with a multistage sampling technique. Four states; Northern, River Nile, Khartoum, and Darfur were randomly selected from the whole country. Then from each state, four localities were selected. Finally, animals were investigated by visiting markets, farms and villages.

b) Collection of blood samples

The blood was taken aseptically from the jugular vein into sterile vacutainers without anti coagulants, allowed to clot for 1-2 hours at room temperature, stored vertically overnight at 4°C and then centrifuged at 4000 rpm for 5 minutes. The sera were taken in sterile bijoux bottles and inactivated in the water bath at 56°C for 30 minutes, allowed to cool and then stored at -20°C until assayed in the laboratory.

c) Data collection

A pre-tested structured questionnaire with the primary objective of elucidating the multi-factorial background of AHS was conducted in an interactive manner with every individual owner of horses and donkeys. The format was designed to investigate individual animal characteristics, management, and environmental risk factors.

d) Laboratory procedure

Blood samples were examined at The Central Laboratory, Ministry of Higher Education and Scientific Research, Khartoum. Competitive Enzyme-Linked Immunosorbent Assay (c-ELISA) was used to detect the presence of specific antibodies against the AHS virus in the collected sera samples following manufacturer's protocol.

e) ELISA protocol

i. Preparation of reagents

Washing solution: one part of the concentrate washing solution which provided in the kit was diluted in 24 parts of distilled or deionized water (40 ml of concentrate solution and 960 ml of water) and remained stable at +4°C.

Controls (+ve) and (-ve): were ready to use and didn't need any preparation.

Conjugate and substrate were ready to use and didn't need any preparation.

1. All reagents were brought to room temperature before use.
2. 100 μ l of the positive control was dispensed into two wells and 100 μ l of the negative control into two wells. Sera samples were diluted 1/5 in provided diluents. This step was made directly in the wells of the plate by adding 80 μ l of the diluents and 20 μ l of serum samples and the plate was shaken carefully for homogenization. The plate was covered and incubated for 1 hour at 37°C.

3. Washing steps:

The content of the plate was thrown out by a brusque turnover of the plate to avoid the possible mixture of the content from one well to another.

A volume of 300 μ l of washing solution was dispensed on each well.

The plate was shaken delicately, avoiding the contamination between wells.

The plate was turned over to empty the wells.

The process was repeated five times as indicated on the instructions of the kit.

Before empty the content of the last washing step, the next reagent was ready to use. The plate was not maintained dry more than strictly needed.

After the last step of washing the plate was shaken and turned over on the absorbent filter paper.

4. The plate was washed five times as described in step 3.
5. 100 μ / well of the conjugate was added. Incubated for 30 minutes at 37°C.
6. The plate was washed five times as described in step 3.
7. 100 μ l/well of the substrate solution was dispensed by using a multi-channel pipette. Incubated for 10 minutes at room temperature.
8. 100 μ l well of the stop solution was dispensed and dispensing of bubbles was avoided.
9. The plate was read at 405 nm using spectrophotometer.
10. Validation criteria:

The ELISA test validation was checked for each plate based on two criteria set by the manufacturer for the mean optical density (OD) of the positive and negative control. The OD of the positive control was less

than 0.2 and the OD of the negative control was higher than 1.0.

11. Interpretation

Blocking percentage (BP) of each sample was calculated based on OD value applying the following formula:

$$BP = \frac{OD (-Control) - OD (Sample)}{OD (-Control) - OD (+Control)} \times 100$$

Samples showed BP value lower than 45% were considered to be negative for antibodies of AHS virus. Samples showed BP value higher than 50% were considered as positive for antibodies of AHS virus.

Samples with BP value between 45% and 50% were considered doubtful, and they were retested. If the result is the same, another extraction was made and tested 2 weeks later.

Samples with BP value between 45% and 50% were considered doubtful and they were retested. If the result is the same, another extraction was made and tested 2 weeks later.

ii. Statistical analysis

All data collected were entered into Microsoft excel spreadsheet. For analysis of the data, SPSS version 21 software was used. Data were analyzed descriptively in the first step, using the frequency table and cross tabulation. Then the association of the potential risk factors with the seroprevalence of AHS at the individual level was analyzed using the Chi-square test. The level of significance was set at $P < 0.05$. For the investigation of the association between the seroprevalence in response to individual animal characteristics, management, and environmental potential risk factors, a multivariate analysis was performed in which logistic regression was used. The strength of association between the risk factors and the sero-prevalence of AHS was quantified using the odds ratio (OR) and the level of significance was set at $p \leq 0.05$.

III. RESULTS

a) Prevalence and associated risk factors

In a total of 920 equines (590 horses and 330 donkeys) sampled and examined, the overall seroprevalence of AHS was 72.2%. Within states, the highest seroprevalence of AHS was reported in Khartoum and Darfur states (81%) and (78.8%), respectively. Table 1 shows the univariate analysis of the association of potential risk factors with the seroprevalence of AHS. The table shows the risk factors which have been investigated, number of animals tested, number of animals positive with their percent (%), the value of Chi-square (χ^2) and the P value.

The seroprevalence of AHS showed statistically significant association with state ($\chi^2 = 47.434$, $p < 0.001$), species ($\chi^2 = 50.163$, $p < 0.001$), sex ($\chi^2 =$

26.206, $p < 0.001$), housing ($\chi^2 = 26.477$, $p < 0.001$), vaccination ($\chi^2 = 44.466$, $p < 0.001$), breed ($\chi^2 = 57.256$, $p < 0.001$), the presence of water bodies ($\chi^2 = 26.271$, $p < 0.001$), presence of culicoides ($\chi^2 = 42.658$, $p < 0.001$), presence of ticks ($\chi^2 = 23.608$, $p < 0.001$), activity of animals ($\chi^2 = 41.435$, $p < 0.001$), awareness of owners ($\chi^2 = 25.639$, $p < 0.001$), age ($\chi^2 = 20.186$, $p < 0.001$), health score ($\chi^2 = 12.038$, $p < 0.001$), pregnancy status ($\chi^2 = 3.249$, $p = 0.0355$), and infection with other diseases ($\chi^2 = 14.637$, $p < 0.001$). However, risk factors of presence of other animals and previous infection with AHS did not show statistically significant associations ($p > 0.05$).

The final multivariate model revealed that equines in Khartoum state were almost five times more likely to be sero-positive compared with equines in Northern state (OR = 4.909, $p = 0.017$), local equines were two times and half more likely to be sero-positive compared with cross equines (OR = 2.532, $p = 0.004$), horses were 3.8 times more likely to be sero-positive compared with donkeys (OR = 3.776, $p = 0.017$), equines raised in areas with water bodies were two times more likely to be sero-positive compared with those in dry areas (OR = 2.172, $p = 0.033$), and non-vaccinated equines were 17 times more likely to be sero-positive compared with vaccinated ones (OR = 17.298, $p < 0.001$).

IV. DISCUSSION

The current study indicated an overall seroprevalence of AHS in equines at the study states of 72.2%. This result was higher than those reported in Khartoum and South Darfur states (Elghazali and Ali 2013, Ihsan 2004), different regions of Ethiopia (Tesfaye et al. 2012, Ende et al. 2013, Kassa 2006) and Zimbabwe (Gordon et al. 2013). In contrast, our findings of the seroprevalence of AHS in donkeys was lower than the previous of Abu Elzein et al. (1989) in Khartoum state. The difference in seroprevalence of AHS in the present study and other previous studies could be probably due to differences in season of sampling, geographic location, study methods and diagnostic techniques employed by the investigators.

Out of 15 risk factors that showed significant statistical association ($p \leq 0.05$) with seroprevalence of AHS in the univariate analysis and entered in the multivariate analysis, only five risk factors (state, species, breed, water bodies and vaccination) showed significant statistical associations ($p \leq 0.05$) with seroprevalence of AHS. The results indicated higher seroprevalence in Khartoum state and South Darfur state which characterized by high rainfall, the presence of water bodies and good vegetation with over-abundance of midges, while River Nile state and Northern state were located in semi-desert and desert climate, respectively. The variations in seroprevalence of

AHS at different states in our study were significant ($p \leq 0.05$), and this result was in close agreement with the results reported by Ende et al. (2013) and Tesfaye et al. (2012) in different regions in Ethiopia.

Furthermore, there was a significant variation of seroprevalence between the two species of equines (horses and donkeys). A higher seroprevalence was observed in horses as compared with donkeys, and the difference was statistically significant ($p \leq 0.05$). This finding is in agreement with a couple of previous studies (Alemayehu and Benti, 2009; Yeshitila and Bekele, 2017). Also, by the OIE (2008) reports which stated that among equines horses were the most susceptible to AHS with a mortality rate of 50-95% followed by mules with a mortality rate around 50% and donkeys with a mortality rate of 5-10%. However, our result differed with the findings of other previous studies conducted by Tesfaye et al. (2012) and Ende et al. (2013) in Ethiopia.

The current study further revealed that there is a statistically significant variation ($p \leq 0.05$) of seroprevalence between the local and cross breed. A higher seroprevalence was observed in the local compared with the cross breed. However, this significant difference could be attributed to the fact that the cross horses receive high care by raising them in safe stables that protect them from the infestation of vectors and vaccinated annually against the disease.

Our findings indicated a statistically significant variation between areas with water bodies and dry ($p \leq 0.05$). A higher seroprevalence of AHS was observed in equines raised in areas with water bodies, and this result is in close agreement with the results reported by Coetzer and Guthrie (2004), Demissie (2013) and Radostitis et al. (2007) who explained that AHS endemic areas are more likely to be low lying, flooded by rain water, warm and marsh regions that create favorable environment for multiplication of *Culicoides* and mechanical vectors.

Furthermore, our final multivariate model revealed a statistically significant ($p \leq 0.05$) variation between non-vaccinated and vaccinated equines. A

higher seroprevalence of AHS was observed in non-vaccinated equines. Lack of vaccination is the most strong risk factor (OR =17.298, $P < 0.001$) among the risk factors investigated and found statistically significantly associated with AHS. However, the observed association could be confounded with poor management and lack of knowledge of equines owners about the disease and the importance of vaccination as a protective tool against AHS. Unfortunately, both risk factors were not investigated.

Regarding sex, our findings showed significant variation ($p \leq 0.05$) in the seroprevalence between sexes in the univariate analysis, with a higher seroprevalence in males than in females. This result is in close agreement with the result reported in Ethiopia by Yeshitila and Bekele (2017). However, the risk factor of sex did not remain statistically significant in the final multivariate model.

In the current study, we have used the odds ratio to quantify the strength of association between potential risk factors and the seroprevalence of AHS. To our best knowledge, this is the first study to quantify the strength of association between potential risk factors and AHS in Sudan. However, although the discussed risk factors were statistically significantly associated with AHS in the final multivariate model, it is difficult to consider them as necessarily causally related and should be interpreted in light of the causal criteria that have been proposed by Thrusfield (2005).

V. CONCLUSION

The present study indicated that AHS seroprevalence was highly prevalent in equines in the study states of Sudan. Furthermore, the seroprevalence of AHS was statistically significantly associated with the vaccination status of equines, geographic location of state, species, breed, and presence of water bodies. These identified risk factors should be carefully considered when control strategies for AHS are implemented.

Table 1: Univariate analysis of potential risk factors associated with sero-prevalence of AHS in equines in four states of Sudan

Risk factors	No. tested	No. +ve (%)	χ^2	p-value
State:			47.434	<0.001
Northern	263	150 (57%)		
River Nile	92	63 (68.5%)		
Khartoum	253	209 (81%)		
Southern Darfur	312	242 (78.8%)		
Species:			50.163	<0.001
Horses	590	472 (80%)		
Donkeys	330	198 (58.2%)		
Sex:			26.206	<0.001
Male	587	455 (77.9%)		
Female	333	209 (62.2%)		
Vaccination:			44.466	<0.001
Yes	197	105 (53.3%)		

No	723	559 (77.3%)		
Health Score:			12.038	<0.001
Good	776	523 (69.7%)		
Bad	164	141 (82.9%)		
Housing:			26.477	<0.001
Barn	170	99 (58.6%)		
Backyard	622	482 (77.2%)		
Farm	128	83 (65.4%)		
Age (yrs):			20.186	<0.001
1 – 3 years	319	285 (80.3%)		
4 – 8 years	547	333 (67.8%)		
9– 12 years	54	34 (60.7%)		
> 12 years	18	12 (66.7%)		
Breed:			57.256	<0.001
Local	836	633(75.7%)		
Cross	84	31 (36.9%)		
Pregnancy Status:			3.249	0.0355
Yes	28	16 (57.1%)		
No	892	648 (72.6%)		

Table 1: Continued

Risk factors	No. tested	No. +ve (%)	χ^2	p-value
Presence of water bodies:			26.271	<0.001
Yes				
No	293	369 (79.7%)		
	627	295 (64.6%)		
Presence of culicoides:			42.658	<0.001
Yes	383	451 (79.8%)		
No	537	213 (60%)		
Presence of ticks:			23.608	<0.001
Yes	189	167 (86.1%)		
No	731	497(68.5%)		
Infection with other diseases:			14.637	<0.001
Yes	77	74 (90.2%)		
No	843	590 (70.4%)		
Presence of other animals:			0.944	0.165
Yes	8	7 (87.5%)		
No	912	657 (72%)		
Pervious infection with AHS:			0.095	0.379
Yes	16	11 (68.8%)		
No	904	653(72.2%)		
Activity of equines:			41.435	<0.001
Racing	131	63 (49.2%)		
Back	352	286 (78.6%)		
Cart	437	315 (73.6%)		
Awareness of owners:			25.639	<0.001
Yes	521	408 (78.8%)		
No	399	256 (63.7%)		



Table 2: Multivariate analysis of potential risk factors associated with sero-prevalence of AHS in equines in four states of Sudan

Risk factors	No. tested	No. +ve (%)	OR	95% CI for OR	p-value
State:					
Northern	263	150 (57%)	Ref.		
River Nile	92	63 (68.5%)	1.637	0.986 – 2.719	0.057
Khartoum	253	209 (81%)	4.909	1.329 – 18.133	0.017
Darfur	312	242 (78.8%)	1.349	0.359 – 5.070	0.658
Breed:					
Local	836	633(75.7%)	2.532	1.339 –4.790	0.004
Cross	84	31 (36.9%)	Ref.		
Species:					
Horses	590	472 (80%)	3.776	1.264 – 11.281	0.017
Donkeys	330	198 (58.2%)	Ref.		
Presence of water bodies:					
Yes	463	369 (79.7%)	2.172	1.063 – 4.437	0.033
No	457	295 (64.6%)	Ref.		
Vaccination:					
Yes	197	105 (53.3%)	Ref.		
No	723	559 (77.3%)	17.298	8.673 – 34.501	<0.001

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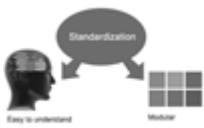
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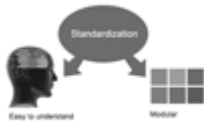
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14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.



20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium through which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

INFORMAL GUIDELINES OF RESEARCH PAPER WRITING

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.



Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.



The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.



Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."



Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

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BY GLOBAL JOURNALS

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Topics	Grades		
	A-B	C-D	E-F
<i>Abstract</i>	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
<i>Introduction</i>	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
<i>Methods and Procedures</i>	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
<i>Result</i>	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
<i>Discussion</i>	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
<i>References</i>	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring



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