

# Immuno-haematological and rheological parameters in canine visceral leishmaniasis

Parâmetros reológicos e imunohematológicos na leishmaniose visceral canina

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Received November 27, 2017

Accepted March 7, 2018

## Abstract

The aim of this study was to find correlations between haematological and rheological parameters in canine visceral leishmaniasis (CLV). Enrolled dogs with CVL (n=31) had a confirmed diagnosis using the rapid test *Dual Path* Platform (TR DPP®) kit and Polymerase Chain Reaction (PCR) assay. A control group (n=31) comprised healthy dogs with negative results for the TR DPP® kit and PCR assay. Haematological parameters and total protein, albumin, globulin, and antibody were assessed. The rheological properties of the blood samples were also determined. Erythrocytes, haematocrit and platelet values of dogs in the CVL group were found to be lower than those of the control group. The total protein, globulin and IgG concentrations were higher in serum samples from the CVL group. The blood flow curve demonstrated increased shear rates in the CVL group. The viscosity of the blood from the infected animals was lower than in the healthy dogs. A positive correlation was found between erythrocyte numbers and blood viscosity. These data suggest that changes in the flow curve and viscosity of blood as well as the erythrocyte values may be a viable low-cost alternative for the monitoring of dogs with visceral leishmaniasis.

**Keywords:** Rheology, *Leishmania chagasi*, dogs, viscosity, erythrocytes.

## Resumo

O objetivo deste estudo foi encontrar correlações entre parâmetros hematológicos e reológicos na leishmaniose visceral canina (LVC). Cães com LVC (n=31) tiveram diagnóstico confirmado de LVC pelo teste rápido imunocromatográfico (TR DPP®) e o ensaio de Reação em Cadeia da Polimerase (PCR). Cães saudáveis negativos para o TR DPP® e PCR foram alocados no grupo controle (n=31). Foram avaliados parâmetros hematológicos, proteínas totais, albumina, globulina, anticorpos e as propriedades reológicas do sangue. Os valores de eritrócitos, hematócrito e plaquetas de cães do grupo LVC foram menores do que os encontrados no grupo controle. As concentrações de proteína total, globulina e imunoglobulina G (IgG) foram maiores em amostras de soro do grupo LVC. A curva do fluxo sanguíneo apresentou maior taxa de cisalhamento no grupo LVC. A viscosidade sanguínea dos animais infectados foi menor do que a viscosidade do sangue dos animais do grupo controle. Uma correlação positiva foi encontrada entre o número de eritrócitos e a viscosidade do sangue. Esses dados sugerem que as mudanças na curva de fluxo e viscosidade do sangue, bem como os valores de eritrócitos, podem ser uma alternativa viável e de baixo custo para o monitoramento de cães com leishmaniose visceral.

**Palavras-chave:** Reologia, *Leishmania chagasi*, cães, viscosidade, eritrócitos.

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## Introduction

Visceral leishmaniasis is a zoonotic parasitic disease caused by a protozoan of the family Trypanosomatidae, genus *Leishmania*. In Brazil, the disease is caused by the species *Leishmania chagasi*, which is transmitted to humans and animals primarily by *Lutzomyia longipalpis* (MISSAWA & LIMA, 2006; MISSAWA et al., 2011; FERREIRA et al., 2012). Visceral leishmaniasis has been identified in all regions of Brazil and due to its high prevalence in dogs, it is believed that they are the primary reservoir in urban areas (MARCHI et al., 2013).

Clinical signs vary from mild to severe. Lymphadenopathy and skin lesions are the most frequently observed symptoms (LIMA et al., 2004; ALMEIDA et al., 2012). Studies have shown the presence of amastigotes in lymph nodes of dogs naturally infected with *L. chagasi* (ALVES et al., 2009). Lymph node hypertrophy / hyperplasia occurs mainly in asymptomatic dogs, whereas atrophy is a predominant characteristic of symptomatic animals (GIUNCHETTI et al., 2008). CVL dogs present lymphadenopathy (LIMA et al., 2004) with inflammatory infiltrates of macrophages and plasma cells, suggesting the importance of innate and humoral immunity (MOREIRA et al., 2010; MOREIRA et al., 2013).

Despite the clinical state, the skin and spleen are the main sites of high parasitic density during the course of CVL (REIS et al., 2009). Anaemia, uraemia, hyperproteinaemia and hyperglobulinemia are common haematological and biochemical abnormalities observed in visceral leishmaniasis. Noteworthy clinical abnormalities include lymphadenopathy, skin changes, anorexia, onychogryphosis, emaciation, pale mucous membranes, ophthalmopathies, fever and diarrhoea (FEITOSA et al., 2000; MAGALHÃES et al., 2012; MENDONÇA et al., 2015).

Techniques that have been used for the diagnosis of leishmaniasis in dogs include the rapid screening test *Dual Path* Platform (DPP®), an ELISA test (BRASIL, 2011), a Polymerase Chain Reaction (PCR) assay and flow cytometry. Although these diagnostic techniques are effective, the high costs of these tests limit their usefulness, thereby creating a need for new tools for the diagnosis and monitoring of this disease.

Laboratory findings such as biochemical and haematological abnormalities have been demonstrated to be of great prognostic value (CIARAMELLA et al., 1997; REIS et al., 2006). In visceral leishmaniasis, anaemia (MENDONÇA et al., 2015) can cause important erythrocytic alterations that likely cause changes in the adhesion and rheological properties of these cells.

It has been demonstrated that blood rheology can be influenced by the viscosity of the plasma, the erythrocytes, as well as factors affecting erythrocyte aggregation (PIAGNERELLI et al., 2003). Infections such as malaria (SCHERER et al., 2016) and diseases such as sickle cell anaemia (HIGGINS et al., 2007) and diabetes mellitus (FRANÇA et al., 2014) have been proposed as disorders in which alterations in blood viscosity may aid in clinical diagnosis.

The correlation between changes in haematological and rheological parameters may be useful as a new method for the diagnosis of visceral leishmaniasis. To investigate this possibility, the present study sought to correlate haematological and rheological parameters in canine visceral leishmaniasis.

## Material and Methods

The procedures developed in this work were approved by the Ethics Committee on Use of Animals-CEUA/UFMT (Approval N°23108.019567/14-1) and the disclosure of results was authorised by the animal owners, who signed an informed consent form.

### Animals

This study was carried out over a 15 month period using 62 dogs of different ages, sexes and breeds, selected and evaluated in the Barra do Garças City, Mato Grosso State, Brazil (Latitude: -15.893, Longitude: 52.2599, 15° 53' 35" South, 52° 15' 36" West). The diagnosis of canine visceral leishmaniasis (n=31) was confirmed in each dog using the rapid test *Dual Path* Platform (RT DPP®) kit and a Polymerase Chain Reaction (PCR) assay. The control group (n=31) comprised healthy dogs without any typical clinical signs of leishmaniasis that had negative results on the RT DPP® kit and PCR assay.

### Blood sampling

Blood samples (3 mL) were collected by cephalic or jugular venipuncture. Portions of each blood sample were placed into tubes without anti-coagulant for obtaining serum as well as into anti-coagulant (Ethylene diamine tetra acetic Acid- EDTA) tubes. The total blood samples were used for rheological and haematological analysis. The serum samples were obtained after centrifugation of the blood sample at 300 x g for 10 min and then transferred to 2 mL microtubes that were stored at -80°C for subsequent measurement of biochemical and immunological parameters.

### Immunochromatographic rapid test-TR DPP® kit

The rapid immunochromatographic tests for detection of anti-*Leishmania chagasi* antibodies (DPP® - Leishmaniasis-visceral-canine-BioManguinhos/FIOCRUZ, Rio de Janeiro, Brazil) were performed according to the manufacturer's protocol.

### Polymerase Chain Reaction – PCR Assay

DNA extraction from blood samples was performed by the phenol-chloroform method (SAMBROOK et al., 1989). The polymerase chain reaction assay was performed using primers RV1(sense) 5'-CTT TTC TGG TCCCGC GGG TAG G-3' and RV2(anti-sense) 5'-CCA CCT GGC TAT TTT ACA CCA-3' (LACHAUD et al., 2002), which amplifies a DNA fragment from a 145bp region of the conserved kDNA present in *L. chagasi*. The amplification utilized 200mM dNTP, 1 pM of each primer, a buffer solution (10 mM of Tris-HCl and 50 mM of KCl, pH 8.3), 2 mM of MgCl<sub>2</sub>, 1.5 U of Taq DNA polymerase and 2µl of the DNA sample in a final volume of 25µl. The assays were run for one cycle at 94°C for 4 min, followed by 30 cycles at 94°C for 30s, 60°C for 30s, 72 °C for 30s, and a one-cycle final step extension at 72 °C for 10 minutes. The amplification product was fractionated

by 2.0% agarose gel electrophoresis, stained with Gel Red stain and visualized in a transilluminator (UV-300nm).

## Haematological parameters

Erythrocytes, total leukocytes and platelet counts were performed as described by Anthony & Sirois (2007). The haemoglobin level was measured by the cyanmethemoglobin method and the haematocrit was determined by the microhaematocrit method. The evaluation criteria and reference values used were those established by Feldman et al. (2000).

### *Albumin, total protein and globulin determination*

The serum biochemistry was assessed with a semiautomatic analyser (Celm SB-190®, São Paulo, Brazil) and commercial kits (Gold Analisa®, Belo Horizonte, Brazil) were used to determine the levels of albumin. Serum total protein was determined by refractometry. The globulin levels were calculated as the difference between the serum total protein and albumin concentrations (THRALL, 2015).

### Immunoglobulin determination

The immunoglobulin (IgM and IgG) concentrations in the sera were determined by the turbidimetric method. For IgM at 1:11 (v/v) and for IgG at 1:15 (v/v), the antibody concentrations were determined using IgM (Bioclin®, Brazil, Ref K063) and IgG (Bioclin®, Brazil, Ref K062) antisera diluted at 1:12 (v/v). A calibration curve obtained from the Multical (Bioclin®, Brazil, Ref K064) calibrator was used to determine the standard curve for each immunoglobulin. Samples of sera, standards, and positive and negative control sera were placed in 500µL of buffer solution (sodium chloride 0.15 mol/L, Tris 50 mmol / L, 6.0000 PEG 50 g/L, and sodium azide 15.38 nmol/L). The suspensions were mixed and incubated at 37°C for 10 min. The reactions were read on a spectrophotometer at 340 nm.

### Blood rheological parameters

The rheological parameters were measured using the Modular Compact Rheometer – MCR 102 [Anton Paar® GmbH, Ostfildern, Germany] as described by França et al. (2014). In all experiments, 600 µL of blood was placed on the surface of a plate reader, followed by the removal of excess sample. The readings were obtained with a permanent control of gap measurements using TruGap™ in 0.099-mm increments and Toolmaster™ CP 50; precise temperature control was achieved using T-Ready™ and Rheoplus V3.61 software. Rheoplus software was also used for the graphics. For the flow curves and viscosity, established parameters were based on the control of shear stress [τ] to 0–5 Pa for the upsweep and 5–0 Pa for downward curves. The tests were conducted under isothermal conditions at 37°C with an analysis of 75 readings.

## Statistical analysis

The data are expressed as the mean ± standard error [SE]. Statistically significant differences between groups were evaluated using Student's t-test. The correlation between immunohaematological parameters and blood viscosity in leishmaniasis was evaluated using the Pearson's linear correlation. Statistical significance was set for p-values lower than 0.05 (P<0.05).

## Results

Among the animals selected for this study, 48.39% were female and 51.61% were male. For the CVL dogs, there was a higher prevalence of males (61.29%) than females (38.71%). The dogs' ages ranged from 1.4 to 8 years in the control group and 1 to 11 years in the CVL group.

Clinical symptoms in seropositive animals (CVL) included lymphadenopathy (77.42%), cutaneous ulcers (58.06%), ear ulceration (51.61%), onychogryphosis (48.39%), desquamation (48.39%), muscular atrophy (35.48%), ophthalmia (35.48%), alopecia (29.03%), splenomegaly (29.03%), lethargy (19.35%), cachexia (30.71%), periocular alopecia (19.35%), hepatomegaly (16.13%), hyperkeratosis (16.13%) and cutaneous nodules (12.90%).

The haematological results are shown in (Table 1). The erythrocytes, haematocrit and platelet values of dogs in the CVL group were found to be lower (p <0.05) than the values for animals in the control group. There were no differences in haemoglobin concentrations or leukocyte counts between the groups studied.

Total protein concentrations and globulin concentrations were higher in the CVL group than in the respective samples from the control group (Table 2). IgG antibody levels were higher in the serum from the CVL group than the control group. Albumin and IgM concentrations were similar between the control and CVL groups.

We evaluated possible correlations between the clinical signs with the haemogram (erythrocytes, haematocrit and platelets). For dogs with CVL, a correlation was observed between erythrocyte numbers and clinical signs of alopecia (r = 0.6762 ; p = 0.0455), splenomegaly (r = 0.9479; p = 0.0001), hepatomegaly (r = 0.8928; p = 0.0414), hyperkeratosis (r = -0.9442; p = 0.0157) and cutaneous nodules (r = -0.9549; p = 0.0114).

**Table 1.** Mean and standard error of erythrocytes, haemoglobin levels, haematocrit, leukocytes and platelets of dogs with canine visceral leishmaniasis (CVL) and control group.

Group	Control	CVL	P*
Erythrocytes (10 <sup>6</sup> /mm <sup>3</sup> )	6.395 ± 0.531	4.545 ± 1.130	0.010
Hemoglobin (g/dL)	15.5 ± 1.394	13.582 ± 3.772	0.064
Haematocrit (%)	45.929 ± 3.653	30.193 ± 7.128	0.010
Leukocytes (10 <sup>3</sup> /mm <sup>3</sup> )	11.189 ± 2.422	9.652 ± 3.849	0.099
Platelets (10 <sup>3</sup> /mm <sup>3</sup> )	266.168 ± 51.816	161.796 ± 41.435	0.010

\*p-value of Student's t-test. P<0.05 significant at 5%.

**Table 2.** Mean and standard error of total protein, albumin, globulin and immunoglobulin (IgG and IgM) levels in the sera of dogs with canine visceral leishmaniasis (CVL) and control group.

Group	Control	CVL	P*
Total Protein	7.364 ± 0.520	9.184 ± 1.339	0.010
Albumin	2.800 ± 0.197	2.360 ± 0.587	0.102
Globulin	3.671 ± 0.482	6.550 ± 1.400	0.010
IgG	636.940 ± 258.033	2300.75 ± 610.071	0.010
IgM	241.120 ± 51.810	279.74 ± 33.990	0.158

\*p-value of Student's t-test. P<0.05 significant at 5%.

The rheological profiles of the blood from dogs with leishmaniasis and from dogs in the control group are shown in (Figure 1). The blood flow curve for both groups begins at the origin, increases, and is nonlinear. An increase in shear rates demonstrated in the blood flow curve of the CVL group (Figure 1).

An analysis of blood viscosity as a function of the shear rates is presented in (Figure 2). The viscosity of the blood from infected animals was observed to be significantly (p = 0.045) lower than for the non-infected animals.

The blood viscosity and rheological parameters were examined for correlations with haematological parameters using the Pearson test (Table 3). A positive correlation (p<0.05) was observed between erythrocyte numbers and blood viscosity. However, no correlation (p>0.05) was found between blood viscosity and the haematocrit, leukocytes, platelets, total protein concentrations, globulin levels or antibody concentrations.

## Discussion

The findings of the present study indicate that animals with visceral leishmaniasis exhibit changes in their blood flow curves and blood viscosity and that the alterations of these rheological parameters are correlated with haematological abnormalities.

Visceral leishmaniasis, which is the most severe form of leishmaniasis, is endemic in more than 80 countries (WHO, 2016). Transmission of leishmaniasis is believed to be increasing in Brazil, with 43% of the population at risk for developing the disease. The high prevalence of infected dogs is an important factor that contributes to the maintenance and transmission of this parasite in urban areas (MARCHI et al., 2013).

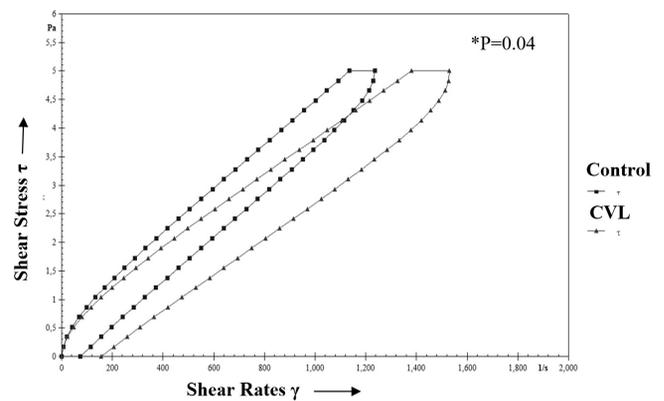
In this study, animals with visceral leishmaniasis demonstrated clinical signs of this disease such as lymphadenopathy, skin ulcers, ulcerated ears, and peeling, onychogryphosis described in previous studies (FEITOSA et al., 2000; MATTOS et al., 2004; MAGALHÃES et al., 2012; ALMEIDA et al., 2012).

Structural changes in erythrocytes and alterations in viscosity have been reported in other diseases (HIGGINS et al., 2007; FRANÇA et al., 2014; SCHERER et al., 2016). In this study, there was a reduction in erythrocyte numbers, the haematocrit and platelet numbers in the blood of dogs with visceral leishmaniasis, indicating mild anaemia (MATTOS et al., 2004; IKEDA-GARCIA & FEITOSA, 2006; MEDEIROS et al., 2008; MENDONÇA et al., 2015) and the likely presence of immunoglobulins capable of binding platelets (TERRAZZANO et al., 2006; CORTESE et al., 2009).

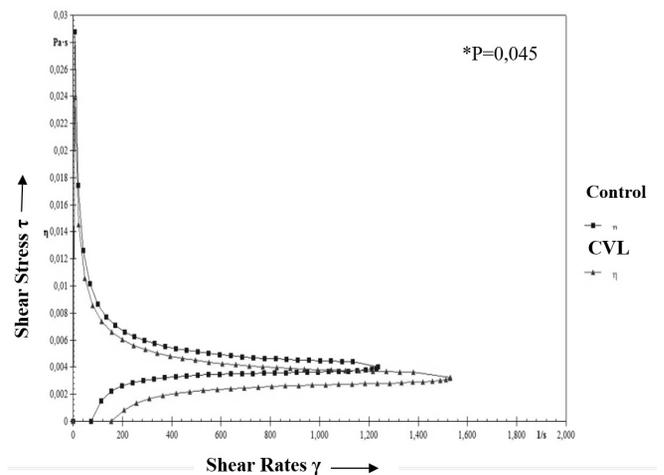
**Table 3.** Correlations between blood viscosity with haematological parameters, proteins and antibody concentrations in the blood of dogs with canine visceral leishmaniasis (CVL) and control group.

Parameters	Viscosity			
	Control		CVL	
	r <sup>a</sup>	P*	r <sup>a</sup>	P*
Erythrocytes	0.0946	0.6126	0.5551	0.0012
Hematocrit	0.2306	0.2120	-0.0570	0.7606
Leukocytes	0.1676	0.3675	-0.0883	0.6368
Platelets	0.0880	0.6377	0.1319	0.5120
Total Protein	-0.0743	0.6913	0.1533	0.4102
Globulins	0.1480	0.7266	0.2282	0.2169
IgG	0.0310	0.9368	-0.1490	0.7248
IgM	0.2005	0.6050	-0.1733	0.6814

<sup>a</sup>correlation coefficient. \*p-value of Pearson's linear correlation test. P<0.05 significant at 5%.



**Figure 1.** Blood flow curves of animals with canine visceral leishmaniasis (CVL) and a control group. These data represent the mean of 31 animals in each group. The data were evaluated by Student's t-test. \*indicates intergroup differences.



**Figure 2.** Blood viscosity curve as a function of shear rates from dogs with canine visceral leishmaniasis (CVL) and a control group. These data represent the mean of 31 animals in each group. The data were evaluated by Student's t-test. \*indicates intergroup differences.

Erythropoiesis may occur due to chronic character, blood loss and lysis of erythrocytes by production of autoantibodies leading to splenic sequestration (TERRAZZANO et al., 2006; IKEDA-GARCIA & FEITOSA, 2006). Thrombocytopenia of infected animals may be associated with the clinical stage of the disease and the presence of IgM and IgG anti-platelet antibodies (CIARAMELLA et al., 2005; TERRAZZANO et al., 2006; CORTESE et al., 2009).

The response to natural infection of *L. infantum* is associated with the presence of IgG (QUINNELL et al., 2003) and IgM specific for *Leishmania* (RODRÍGUEZ et al., 2006). Some studies have reported that an increase in total protein occurs frequently in dogs infected with visceral leishmaniasis due to the increased production of antibodies (CIARAMELLA et al., 1997; MEDEIROS et al., 2008). This study reinforces the hypothesis that increases in total protein and the globulin fraction in the serum of dogs with visceral leishmaniasis is likely associated with higher concentrations of IgG.

Serum albumin concentrations were similar between the two groups of dogs. Studies report that hypoalbuminemia and hyperglobulinemia (FREITAS et al., 2012) and albumin/globulin inversion (MENDONÇA et al., 2015) are frequent changes in dogs with visceral leishmaniasis and suggest that the inversion of these proteins may occur due to the increase in the production of antibodies to *Leishmania* spp. (SILVA et al., 2008).

Alterations in erythrocytes, inhibition of normal erythrocyte aggregation (PIAGNERELLI et al., 2003), increases in glucose levels (FRANÇA et al., 2014), protein levels (FORESTO et al., 2005) and infections (SCHERER et al., 2016) may influence blood viscosity.

The rheological properties of erythrocytes are important gauges, as they may indicate increased destruction of these cells, leading to anaemia (MOHANDAS & AN, 2012). In this study, dogs with visceral leishmaniasis had lower blood viscosities and lower percentages of erythrocytes and haematocrits than the controls, suggesting that anaemia may be associated with rheological alterations. Studies report that haematocrit values above 50% cause an increase in viscosity, while values below 35% lead to a reduction in blood viscosity (MARTINS & SILVA, 1983). It should be noted that the viscosity is directly associated with resistance to blood flow and the shear stress rates in the vascular wall (YEOM et al., 2014).

The rheological profile of the blood flow, independent of infection, demonstrates a blood flow curve that begins at the origin. Ascending and non-linear behaviour that starts at the origin characterizes non-Newtonian and pseudoplastic fluids under these shear conditions (ROSENCRANZ & BOGEN, 2006; VERDIER et al., 2009).

In the ascending and descending flows, the upper curve is not superimposed on the lower curve; between them is an area of hysteresis, which defines the magnitude of the property of thixotropy (FRANÇA et al., 2014). The presence of a hysteresis area was observed in our study, suggesting that thixotropic properties are present in the blood stream of animals infected with *Leishmania chagasi*. However, the blood flow curve in this group demonstrated an increase in the shear rate, creating a deviation of the flow curve to the right.

The rheological behaviour of blood is influenced by the properties and interactions of the cellular components and plasma (MARTINS & SILVA, 1983). *Leishmania* spp. are obligatory intracellular organisms of the mononuclear phagocytic system (CORTESE et al., 2012). Leukocytes are also rheologically important for flow at the microcirculation level (ERNST & BERGMANN, 1989).

In high concentrations, leukocytes and platelets may become physical obstacles to erythrocyte microcirculation. Temporary or permanent occlusions of the bloodstream may occur if there is reduced deformability of the red blood cells or blood pressure (MARTINS & SILVA, 1983). Although in this study no leukocyte alterations were observed, further studies should be performed to demonstrate the role of these cells during *Leishmania chagasi* infection.

The rheological changes caused by viscosity reduction and increased shear rates associated with decreased erythrocytes and haematocrit levels may be useful as a new tool for the monitoring of animals with visceral leishmaniasis.

Interestingly, blood viscosity and erythrocyte numbers were positively correlated. These findings demonstrate that a reduction in erythrocytes also reduces viscosity and confirm the importance of erythrocytes and blood viscosity changes in leishmaniasis infection.

These data suggest that the effects of leishmaniasis on the flow curve and viscosity of blood in association with erythrocyte values may be considered to be a viable, low-cost alternative for monitoring of dogs with visceral leishmaniasis.

## Acknowledgements

This research received grants from Fundação de Amparo à Pesquisa de Mato Grosso (FAPEMAT N° 299032/2010) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq N° 447218/2014-0 N°: 308600/2015-0), Brazil.

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