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Research Article

Attenuated blood coagulation in wistar rats fed graded levels of a protein diet

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ABSTRACT

Background: Blood coagulation (haemostasis) is a defense mechanism that helps to prevent the excessive loss of blood from damaged blood vessels by formation of a plug. The relationship between diet and blood coagulation has been studied over time. However, there is a dearth of information regarding the effect of consumption of high levels of casein and crayfish proteins on blood coagulation. **Aim:** The purpose of this study was to evaluate the impact of consumption of graded levels of casein and crayfish proteins on blood coagulation in Wistar rats. **Method:** Graded levels of casein (milk protein) and crayfish (seafood) were incorporated in rat diet as sources of protein at 20%, 30% and 50% concentrations. At the end of the experimental period (28 days), Platelet Count, Bleeding Time, Clotting Time, Prothrombin Time, Activated Partial Thromboplastin Time, and Fibrinogen Concentration were evaluated. Standard data analytical software (Graphpad prism version 5.0) was used to analyze the data. **Results:** From the findings of this study, when compared with the control, platelet count and fibrinogen concentration were significantly reduced ($p<0.05$) at 50% concentration while clotting time, Prothrombin Time and and Activated Partial Thromboplastin Time were elongated significantly ($p<0.05$) at 50% concentration; the elongation of the BT was not statistically significant ($P>0.05$). **Conclusion:** Consumption of high concentration of casein and crayfish proteins consistently over a period can attenuate blood coagulation and may predispose to excessive bleeding, with possible adverse cardiovascular consequences.

INTRODUCTION

Protein is an indispensable macronutrient needed by the body in adequate amount for development and maintenance (Berg *et al.,* 2002). Despite its importance, research findings suggest that consumption above the required amount may have some adverse effects on adults (Delimaris, 2013). Reports by Bilsborough and Mann, 2006, opined that there are some situations that may require people to consume greater or lesser amount of protein than the average person. Consumption of dietary protein more than the required amount may be required in certain conditions such as in growing children (Uauy *et al.,* 2015), in the elderly (Wolfe *et al.,* 2008), in medical conditions such as sarcopenia (Paddon-Jones *et al.,* 2008), in athletes (Phillips and Van Loon, 2011) and for weight loss (Magkos, 2020). Low protein regime has been suggested in kidney and liver dysfunctions (Hoffer and Bistrian, 2012; Ko *et al.,* 2017). There are however conflicting reports in literature of the effects of consumption especially of animal proteins on some cardiometabolic diseases (Petersen *et al.,* 2017).

Crayfish is one of the most common seafoods consumed worldwide. It is a rich source of protein, B vitamins, iron, and selenium. In Nigeria, it is widely eaten and used in the preparation of dishes (Anthonio and Isoun, 1982). Casein is the main protein of milk which accounts for about 80% of the total protein. It is widely consumed by athletes, as it has been reported to be more effective in stimulating amino acids uptake and net protein deposition in skeletal muscles after resistance training (Phillips *et al.,* 2009).

Haemostasis, one of the defense mechanisms of the body, is necessary for survival as it helps to stop excessive bleeding (Verhamme and Hoylaerts, 2009). Findings in literature clearly indicate that some

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abnormal haemostatic states can be prevented or treated with diet (Volster *et al.*, 1997). Valluanou *et al.*, 2013, reported a reduction in blood platelet count in human subjects following consumption of high concentration of plant protein. According to Chang *et al.*, (1997), low protein diet resulted in blood coagulation deficit in experimental rats. Prolonged activity of the intrinsic coagulation pathway was reported in sardine protein fed experimental rats (Murata *et al.*, 2004). Flavonoids found in chocolates has been reported by Coakley *et al.*, (2005), to have antiplatelet effect, while some other researchers have suggested that consumption of alcohol may result in hypo-coagulation in men (Spoerke *et al.*, 2010). Some vegetarian diets have been shown to increase platelet aggregation (Mezzano *et al.*, 1999; Sujaya, 2003). Song *et al*, (2016) opined that excessive intake of proteins can be associated with high mortality rate.

The dearth of literature on the effects of consumption of high levels of casein and crayfish on blood coagulation necessitated this study which evaluated the possible attenuated blood coagulation in Wistar rats fed graded levels of a protein diet (casein and crayfish proteins).

METHODS

Forty-nine (49) 12-week-old adult male albino Wistar rats were used for this study. The rats weighing between 180-200g were obtained from the Animal House of the Department of Anatomy, University of Benin, Benin city Edo State Nigeria. The rats were kept in cohorts and housed in standard cages constructed with wood and wire mesh and allowed to acclimatize for two (2) weeks, thereafter, weighed and randomly assigned to control and experimental groups. Group 1 (Control) were fed with normal rat chow (14% protein concentration) and water, Group 2 were fed with ration containing graded levels of casein as source of protein. Based on protein concentration, this group was further divided into three subgroups namely: 2a (20%), 2b (30%) and 2c (50%). Group 3 were fed with feed ration containing graded levels of crayfish as source of protein. Based on concentration, this was further divided into 3a (20%), 3b (30%) and 3c (50%), thus, making a total of seven groups. The experimental period was twenty-eight (28) days. All the rats were supplied with feed and water *ad libitum*.

Sample Collection

At the end the experimental period (28 days) the rats (control and experimental) were restrained, then bleeding time and clotting time investigations were carried out. Thereafter, the rats were then sacrificed via cervical dislocation. Blood samples were collected via cardiac puncture into blood sample containers containing 3.2% sodium citrate in a ratio of 1:9: that is one part anticoagulant to nine parts of blood.

Bleeding Time

Bleeding Time assessment was done using Duke's method modified by Ochei and Kolhatkar (2000). Using sterile lancet, two skin punctures were made quickly on the tail of the rat. The timer (stopwatch) was immediately started. The puncture sites were dabbed with Whatman No 1 filter paper every 15 seconds until there was no more blood stains on the filter paper. The time of stoppage of blood was recorded as the bleeding time.

Clotting time

Clotting time assessment was done using capillary glass tube method (Harris et al., 1956). From the tail of the rat, blood was collected into plain (non-heparinized) capillary tubes, and the timer was started immediately. From one end of a capillary tube (one capillary tube at a time) containing the blood, pieces of capillary tube glass were broken off and pulled apart gently at 30 seconds interval while watching out for the appearance of fibrin strands. The timer was stopped as soon as fibrin threads appeared as this served as the end point (clotting time) and the time was noted in seconds.

Platelet Count

Determination of platelet count was carried out using automated method. ERMA PCE 210 Haematology Analyzer.

Fibrinogen Concentration

Plasma fibrinogen concentration was determined using the clot weight method of Ingram (1961). Blood was first collected into sample containers containing 3.2% sodium citrate in the ratio 1:9 with blood. Blood plasma was obtained by centrifuging blood in a stoppered vial at 1000 g for 10 minutes. 0.2 ml of the test plasma was put into a test tube and incubated in a water bath for 3 minutes at 37°C. 0.2 ml of thrombin time reagent was added to the test plasma, mixed and the clot formed harvested with a wooden applicator stick. The resulting clot was transferred into a tube containing acetone to dry and harden for about 10 minutes; the acetone was decanted, and the clot placed on a filter paper for the remaining acetone to evaporate. The clot was then recovered and weighed. The process of fibrinogen concentration determination was completed within 3 hours of blood collection. The fibrinogen concentration

of the citrated plasma in mg/dl was computed as clot weight (mg) divided by plasma volume (dl).

Prothrombin Time

Clotting tubes were prewarmed in a water bath at 37^0C before the commencement of the experiment. 100 µl of citrated plasma was dispensed into a tube, transferred into the water bath and incubated for two minutes. 200 µl of calcium rabbit brain thromboplastin was dispensed into the tube containing the citrated plasma in the water bath. This was mixed and the stopwatch started immediately. Periodically, prior to the estimated coagulation time, the tube was removed from the bath and tilted by gently sliding the liquid content along the length of the clotting tube from the bottom to the middle while watching out for the appearance of a clot. The tube was always returned to the water bath immediately every time. The time for the first appearance of clot was recorded in seconds. The procedure was repeated for control sample.

Activated Partial Thromboplastin Time (APTT) assay

Prior to testing, all reagents, controls, and samples were brought to room temperature for 15 minutes. 100 µl of PTT reagent (kaolin platelet substitute mixture) was dispensed into a clotting tube in a water bath. The tubes were tilted at regular intervals for 2 minutes. 100 µl of calcium chloride was added into the tube and stop watch was started. The tubes were tilted at regular intervals and returned to the water bath while watching out for clot. The time for the first appearance of clot was recorded. The procedure was repeated for control sample. The result was compared with manufacturer's range. Periodically, prior to the estimated coagulation time, the tube was removed from the bath with a gentle sliding of the liquid content along its length from the bottom to the middle whilst watching out for the appearance of a clot in each of the tube.

Statistical Analyses

Standard data analytical software (Graphpad prism version 5.0) was used to analyze the data. Results obtained were expressed as mean ± SEM. One-way ANOVA was employed. Post Hoc using Tukey's least significant differences (LSD) showed statistically significant difference and $P < 0.05$ was regarded as statistically significant.

RESULTS

The effects of consumption of graded levels of casein and crayfish proteins on blood coagulation parameters were investigated. The results showed that there was a concentration dependent effect of the graded protein rations on the coagulation parameters of the experimental rats. The results are as displayed in tables 1 and 2 below.

Bleeding Time (BT)

From the findings of this work, BT was elongated in both the casein and crayfish fed rats which was not statistically significant ($p>0.05$) when compared with the control (Tables 1 and 2). The highest record was in the rats fed with 50% (casein: 10.8±2.53, crayfish: 10.3±1.61**)** when compared with the control (8.2±1.02).

Clotting Time (CT)

The results showed elongation of the CT with increase in protein concentration (Tables 1 and 2). Statistically significant increased results were recorded in the 50% concentration in casein (196±4.66**) and crayfish (188±4.61**) when compared with the control (128±4.18). The result of the lesser concentrations of both casein and crayfish fed rats were however not statistically significant

Platelet Count (PC)

The results showed a reduction in the PC with increase in protein concentration (Tables 1 and 2). Statistically significant decreases were recorded in the 50% concentration in casein (329±7.31**) and crayfish (368±9.87*) when compared with the control (483±6.77). The results of the lesser concentrations of both proteins were not statistically significant

Fibrinogen Concentration (FC)

The results showed a reduction in the FC with increase in protein concentration (Tables 1 and 2). When compared with the control, statistically significant decrease was recorded in the 50% (139.1±4.71**) casein concentration. Also, 30 % (156.6±4.67*) and 50 % (142.3±5.4**) crayfish concentrations were observed to be significantly reduced when compared with the control (189±6.73).

Prothrombin Time (PT)

The results showed a reduction in the PT with increase in protein concentration (Tables 1 and 2). Statistically significant increased results were recorded in the 50% concentration of casein (22.4±1.81*) and crayfish (20.8±2.02*), when compared with the control (13.4±2.30). The increases in the lesser concentrations were not statistically significant.

Activated Thromboplastin Time Test (APTT)

The results showed an elongation in the APTT with increase in protein concentration (Tables 1 and 2).

Statistically significant increases were recorded in the 50% concentration of casein (29.1±1.04*) and crayfish (**28.9±1.98***), however, the result of the 20 and 30 % casein and crayfish fed rats were not statistically

Table 1: Effect of graded concentrations of casein on Coagulation parameters

Protein Source	Protein Conc.	PC (10^3/µL	PT (s)	APTT (s)	BT (m)	CT (s)	FC (mg/dl)
Control	14%	483±6.77	13.4±2.30	17.8± 1.72	8.2±1.02	128±4.18	189.6±6.73
Casein	20%	461±8.90	13.9±1.05	18.2±2.98	7.5±0.95	121±4.62	179.3±5.22
Casein	30%	425±6.98	17.6±2.50	22.1±3.17	9.4±1.67	141±2.66	168.2±6.54
Casein	50%	329±7.31**	22.4±1.81*	29.1±1.04*	10.8±2.53	196±4.66**	139.1±4.71**

Key: PC- Platelet Count; PT- Prothrombin Time; APTT- Activated Partial Thromboplastin Time; BT- Bleeding Time; CT- Clotting Time; FC- Fibrinogen Concentration; s- seconds; m- minutes. Values are expressed as means ± SEM, n = 7. * Difference from control is statistically significant at $P < 0.05$.

Table 2: Effect of the graded concentration of Crayfish on Coagulation parameters

Protein Source	Protein Conc.	PC (10^3/µL	P T (s)	APTT (s)	BT (m)	CT (s)	FC (mg/dl)
Control	14%	483±6.77	13.4±2.30	17.8± 1.72	8.2±1.02	128±4.18	189.6±6.73
Crayfish	20%	509±9.60	14.3±3.76	18.8±2.02	8.1±2.11	126±3.70	198.4±5.12
Crayfish	30%	405±.01	18.7±3.43	23.5±2.31	9.7±1.98	152±3.88	156.6±4.67*
Crayfish	50%	368±9.87*	20.8±2.02*	28.9±1.98*	10.3±1.61	188±4.61**	142.3±5.4**

Key: PC- Platelet Count; PT- Prothrombin Time; APTT- Activated Partial Thromboplastin Time; BT- Bleeding Time; CT- Clotting Time; FC- Fibrinogen Concentration; s- seconds; m- minutes. Values are expressed as means ± SEM, n = 7. * Difference from control is statistically significant at $P < 0.05$.

significant when compared with the control (17.8±1.72).

DISCUSSION

This present study was designed to investigate the possible debilitating effects of the consumption of high concentrations of casein and crayfish proteins on blood coagulation of Wistar rats. Blood coagulation is an important physiological function (Martini *et al.*, 2016), failure of which can lead to coagulation disorder, and possible uncontrolled blood loss and death (Gonzalez *et al.*, 2014).

As observed in this study, the consumption of high levels (50%) of casein and crayfish proteins predisposed to dissipated blood coagulation when the results of the parameters of the experimental rats were compared with the control. The reduction in the platelet counts (thrombocytopenia) with corresponding increase in protein concentration clearly suggested a compromise in the aggregating ability of the platelet and hence an impairment of platelet function. Prior findings by Akoh and Hearnberger (1991), described a similar observation of a reduction in platelet count resulting from consumption of salmon-rich diet. During injury, platelets help to reduce blood loss by forming a plug and promoting tissue repair (Nurden *et al.*, 2008). A reduction in fibrinogen concentrations (hypofibrinogenaemia) with increase in protein concentration was also observed in this study, further buttressing the attenuating effect of casein and crayfish proteins at high concentrations on blood coagulation over a period of time. In hypofibrinogenaemia (Factor I deficiency), the formation of fibrin and stable clot is impaired. The unstable clot so formed breaks down in vivo resulting in some cardiovascular challenges. Low fibrinogen has been reportedly observed in patients with reduced synthesis due to hepatic impairment or haemodilution (Besser and MacDonald, 2016). Bleeding time, a test which helps to monitor platelet function was observed to be unduly prolonged, which further validates the thrombocytopenia observed in this study. Clotting time finding which was significantly prolonged in this study also pointed to a likely predisposition to excessive bleeding.

Prothrombin (PT) and activated partial thromboplastin time tests (APTT) are basic coagulation tests which

measure integrated actions of majority of coagulation factors in extrinsic and intrinsic pathways of coagulation cascade of blood (Abdollah *et al.,* 2013). The activities of these pathways were observed to be significantly reduced in the experimental rats fed with the highest concentration of the experimental protein diets when compared with the control. These observations were in line with investigations by Murata *et al.*, (2004) and Yoko *et al.*, (2003) who reported prolonged APTT and PT in rats fed high concentration of sardine protein, and high fish oil content. Prothrombin time is an important test which helps to check the presence of five different blood clotting factors (factors I, II, V, VII, and X). Coagulation factors are produced by the liver (Heinz and Braspenning, 2015) therefore the coagulation deficit observed in this study may be a reflection of an incapacitation of the liver in performing its synthetic role. The consumption of excessive quantity of casein and crayfish proteins may have imposed a metabolic burden on the liver hence limiting it in effectively synthesizing the coagulation factors as already pointed out by some researchers (; Delimaris, 2013: Diaz-Rua et al., 2017).

We hereby conclude from the findings of this study that, consumption of high levels of crayfish and casein proteins resulted in hypofibrinogenaemia as well as impairment of platelet function. Also, the basic coagulation tests which measure integrated actions of majority of coagulation factors in extrinsic and intrinsic pathways of coagulation cascade of blood were attenuated.

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REFERENCES

Abdollahi A., Shoar N., Shoar S., and Rasoulinejad M. (2013). Extrinsic and intrinsic coagulation pathway, fibrinogen serum level and platelet count in HIV positive patients. *Acta Med Iran*. **51**(7):472-6.

Akoh, C.C. (Alabama A and M University, Normal, AL); Hernsberger, J.O. (1991) Effect of catfish and salmon diet on platelet phospholipid and blood clotting in healthy *men. The* journal of nutritional biochemistry (USA)*ISSN:* 0955-2863 **2**(6)329-333

Anthonio H.O., Isoun, M. (1982). Nigerian Cookbook. Macmillan, London. ISBN 13: 9780333326985

Berg J. M., Tymoczko J. L., and Stryer L. (2002). Protein Structure and Function. Biochemistry 5th Edition, New York: WH Freeman; Chapter3,

Besser, M. W., and MacDonald, S. G. (2016). Acquired hypofibrinogenemia: current perspectives. *Journal of blood medicine*, **7**: 217–225.

Bilsborough S., and Mann N. (2006). A review of issues of dietary protein intake in humans. *International Journal of Sport Nutrition and Exercise Metabolism. **16**(2):129–52.*

Diaz-Rua R., Keijer J., Palou A., van Schothorst E. M., and Oliver P. (2017) Long-term intake of a high-protein diet increases liver triacyglycerol deposition pathways and hepatic signs of injury in rats. *J Nutr Biochem.* **46**:39-48

Song, M., Fung, T. T., Hu, F. B., Willett, W. C., Longo, V. D., Chan, A. T., & Giovannucci, E. L. (2016). Association of Animal and Plant Protein Intake With All-Cause and Cause-Specific Mortality. *JAMA internal medicine*, **176**(10), 1453–1463.

Gonzalez E., Moore E. E., Moore H. B., Chapman M. P., Silliman C. C., and Banerjee A. (2014). Trauma-Induced Coagulopathy: An Institution's 35 Year Perspective on Practice and Research. *Scandinavian Journal of Surgery*. **103**(2):89-103.

Heinz S. and Braspenning J. (2015). Measurement of Blood Coagulation Factor Synthesis in Cultures of Human Hepatocytes Methods. *Mol Biol* **1250**:309-16

Ko G. J., Obi Y., Tortoricci AR. and Kalantar-Zadeh K. (2017). Dietary Protein intake and Chronic Kidney Disease. *Curr Opin Clin Nutr Metab Care* **20**(1):77-85

Magkos F. (2020). The role of dietary Protein in obesity. *Rev Metab Disord*. **21**(3):326-340

Martini W. Z. (2016). Coagulation complications following trauma. *Military Medical Research*, **3:** 35.

Mezzano, D., Muñoz, X., Martínez, C., Cuevas, A., Panes, O., Aranda, E., Guasch, V., Strobel, P., Muñoz, B., Rodríguez, S., Pereira, J., & Leighton, F. (1999). Vegetarians and cardiovascular risk factors: hemostasis, inflammatory markers and plasma homocysteine. *Thrombosis and haemostasis*, *81*(6), 913–917.

Murata M., Sano Y., Bannai S., Ishihara K., Matsushima R. and Uchida M. (2004) Fish protein stimulated the fibrinolysis in rats. *Ann Nutr Metab*. **48**(5):348-56.

Nurden A.T., Nurden P., Sanchez M., Andia I. and Anitua E. (2008). Platelets and Wound Healing. *Front Biosci.* **1**(13):3532-48

Paddon-Jones D., Campbell W. W., Volpi E. and Wolfe R. R. (2008). Role of dietary protein in the

sarcopenia of aging. *Am J Clin Nutr*. **87**(5):1562S-1566S.

Petersen K. S., Flock M. R., Ritcher C. K., Mukherjea R., Slavin JL. and Kris-Etherton P. M (2017). Healthy Dietary Patterns for Preventing Cardiometabolic Disease: The Role of Plant-Based Foods and Animal Products. *Curr Dev Nutr*. **1**(12)

Phillips S. M and Van Loon L. J. (2011). Dietary protein for athletes: from requirements to optimum adaptation. *J Sports Sci.*29 Suppl **1**: S29-38.

Phillips S. M., Tang J. E., Moore D. R. (2009). The role of milk- and soy-based protein in support of muscle protein synthesis and muscle protein accretion in young and elderly persons. *J Am Coll Nutr*. **28**(4):343-54.

Spoerke N., Underwood S., Differding J., Van P., Sambasivan C., Shapiro D. and Schreiber M. (2010) Effects of ethanol intoxication and gender on blood coagulation. *J Trauma*. **68**(5):1106-11.

Uauy R., Kurpad A., Tano-Debrah K., Otoo G. E, Toride Y. and Ghosh S. (2015). Role of Protein and AminoAcids in infant and Young Child Nutrition: Portein and Amino Acid Needs and Relationship with Child Growth. *J Nutri Sci Vitaminol*. **61** suppl: S192-4

Vallianou, N. G., Bountziouka, V. P., Georgousopoulou, E., Evangelopoulos, A. A., Bonou, M. S., Vogiatzakis, E. D., Barbetseas, J. D., Avgerinos, P. C. and Panagiotakos, D. B. (2013). Influence of protein intake from haem and non-haem animals and plant origin on inflammatory biomarkers among apparently-healthy adults in Greece. *Journal of health, population, and nutrition*, **31**(4), 446–454.

Verhamme P. andHoylaerts MF. (2009). Haemostasis and Inflammation: two of a kind? *Thrombosis J*. **7**(15)

Yow-Ling C., Hee-Sook S., Kung-Chi C., Carolyn D. B., James L. H., (1997). Low Dietary Protein Impairs Blood Coagulation in BHE/cdb Rats, *The Journal of Nutrition*, **127**(7):1279–1283,

Hoffer L. J. and Bistrian B. R. (2012) Appropriate protein provision in critical illness: a systematic and narrative review. *Am J Clin Nutr*. **96**(3):591-600.

Wolfe R. R., Miller S. L. and Miller K. B. (2008) Optimal protein intake in the elderly. *Clin. Nutr*. **27**:675–684.

Vorster H. H., Cummings J. H. and Veldman F. J. (1997) Diet and haemostasis: time for nutrition science to get more involved British Journal of Nutrition. **77**:671-684