Original Article

Effects of Exogenous Neurokinin B Administration on Hematological and Coagulation Parameters in Adult Male New Zealand White Rabbits

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Abstract

Objective: To explore the effects of varying doses of Neurokinin B (NKB) on hematological and coagulation parameters in adult male rabbits.

Methodology: This experimental study was conducted at Gomal University, Dera Ismail Khan, Pakistan from June-2019 to June-2020. Eighteen adult male New Zealand white rabbits, weighing 1.5–2 kgs each, were divided randomly in to three groups (n=6 animals/group). Group-I rabbits (control) received injections of distilled water subcutaneously. Group-II rabbits received 1 µg NKB, Group-III rabbits received 1ηg NKB subcutaneously twice daily (12 hourly), consecutively for 12 days. Animals from each group were sacrificed after 12 days of continuous treatment and blood collected for analysis of hematological [complete blood count (CBC)] and coagulation parameters [Bleeding time (BT), clotting time (CT), prothrombin time (PT) and activated partial thromboplastin time (APTT)]; CBC was performed by automated hematology analyzer, while CT and BT by Duke's and capillary tube methods respectively; PT and APTT were analyzed using commercially available kits as per manufacturer's instructions.

Results: BT was significantly prolonged in NKB-treated rabbits compared to control (group-II: 367.5±28.06sec, group-III: 302.5±39.59sec, control: 102.5±22.08sec). CT was also increased (group-II: 317.50±25.84sec, group-III: 280±32.40sec, control: 157.50±28.06sec), as did PT (group-II: 37.0±3.69sec, group-III: 23.67±1.21sec, control: 22.0±1.41sec) and APTT (group-II: 39.0±2.89sec, group-III: 25.50±1.87sec, control: 20.83±2.32sec). Additionally, mean platelet volume [group-II: 6.57±0.30fl, group-III: 6.55±0.36fl, control: 6.05±0.27fl] and leukocyte count (×103/µl) [group-II: 15.73±1.38, group-III: 14.88±1.56, control: 8.22±1.16] were significantly increased in NKB-treated animals. International normalized ratio was significantly increased at group-II (2.0±0.1) compared to control (1.1±0.07). Conversely, platelet count (×103/µl) was decreased significantly in all NKB-treated animals (group-II =143.50±13.60, group-III=179.67±28.09) compared to control (246.34±28.73).

Conclusion: Neurokinin B administration led to leukocytosis, thrombocytopenia, with prolonged BT, CT, PT and APTT dose-dependently.

Keywords: Tachykinins, Neurokinin B, Platelets, Blood Coagulation, Activated Partial Thromboplastin Time, Prothrombin Time, Blood Coagulation Tests.

Introduction

Neurokinin B is a fascinating decapeptide and belongs to a family of peptide hormones known as tachykinins.1 They play vital roles in the body, affecting various tissues and organs. The mammalian tachykinin family includes six key peptides: Neurokinin A, Neurokinin B, Neuropeptide K, Neuropeptide γ, Substance P (SP), and Hemokinin-1.[1] It is interesting to observe that inspite of such diversity in their functions, all of them share a common structural sequence of five amino acids (Phe-X-Gly-Leu-Met-NH2), which makes them unique and researchable.[1] Tachykinins exert their biological effects through three specific G-protein coupled receptors: NK1, NK2, and NK3.[1] Each tachykinin has a preferred receptor—Substance P, which primarily binds to NK1, Neurokinin A to NK2, and Neurokinin B to NK3.[1] However, these peptides can also interact with the other receptors with lower affinity, enabling them to influence a wide range of physiological processes.[1]

Neurokinin B is synthesized from a larger precursor protein encoded by the pre-protachykinin-B gene.[1] This precursor undergoes enzymatic cleavage to form pro-neurokinin B, which is then converted to Neurokinin B.[2] The primary actions of Neurokinin B are mediated through the NK3 receptor, present in both the central nervous system and peripheral tissues.[1]

Evidence for the critical role of Neurokinin B originates from its prominent involvement in reproductive health. Research indicates that mutations in the TAC3 gene, which encodes Neurokinin B, can result in hypogonadotropic hypogonadism, characterized by inadequate production of sex hormones.[3-5] Beyond its role in reproduction, Neurokinin B is involved in a spectrum of biological processes. Its activity has been observed in immune responses, inflammation, and even in conditions such as pre-eclampsia, menopause, and cancer.[2,6-8] Substance P, another tachykinin, can activate immune cells and promote the production of inflammatory molecules.[9] Both Neurokinin B and Substance P are linked to the development of certain cancers, including breast and gastric cancer.[6, 10]

Bleeding disorders, inherited in an autosomal recessive manner, are common worldwide. Pakistan's high rate of consanguineous marriages likely increases the prevalence of conditions like thalassemia, hemophilia, and other coagulation disorders, posing significant public health challenges.[11] However, these disorders often remain undiagnosed due to a lack of specialized diagnostic facilities, low public awareness, and low

clinical suspicion.[11] Insights into how Neurokinin B influences blood cell counts and clotting mechanisms could lead to new therapeutic approaches for managing these conditions.

However, keeping into account its important and diverse involvement in various physiological processes, the effects of Neurokinin B on blood and clotting parameters remain largely unexplored. Blocking NK1 receptors has been shown to reduce platelet aggregation and thrombus size, highlighting the important role of tachykinins in coagulation.[12] This study aims to bridge this gap by investigating how different doses of Neurokinin B affect blood cell counts and clotting factors in adult male rabbits following subcutaneous injection. By exploring these effects, we hope to provide deeper insights into the hematological and coagulation dynamics influenced by tachykinins. The objectives are to quantify changes in white blood cells, red blood cells, platelets, and coagulation parameters, providing new insights into the hematological and coagulation dynamics influenced by Neurokinin B.

Methadology

Animal Handling and Housing

This experimental study was carried out over the course of a year, from June 2019 to June 2020. Eighteen healthy adult male New Zealand white rabbits, each weighing between 1.5 and 2 kg, were acquired from the National Institute of Health, Islamabad. The animals were housed in the animal facility at Gomal Centre of Biochemistry and Biotechnology, Gomal University, Dera Ismail Khan, Pakistan for the duration of the trial. Before the experiment commenced, the animals underwent a 10-day acclimatization period. They were kept under a 12-hour light/dark cycle at a constant temperature of 25±2°C, with free access to food and water. European Union regulations for the use of animals in research were followed for handling the animals during the trial period. The study was approved by the Ethics Review Board of Gomal University vide letter # 118/QEC/GU dated 29–01–2019.

Peptide Dosage and Treatments

Neurokinin B was sourced in lyophilized form from Sigma Aldrich (USA) having catalogue # of N4143-1MG. A stock solution was created by dissolving the peptide in 1ml of dimethyl sulfoxide (VWR, USA). This stock solution was then diluted with distilled to obtain desired concentrations of the peptide for subcutaneous injections. Animals were randomly allocated to different groups (n=6 per group). Group-I served as a control and received subcutaneous injections of distilled water. Group II was administered 1 µg of Neurokinin B subcutaneously, while Group III received 1 ηg of Neurokinin B subcutaneously, both dosages given twice daily at 12-hour intervals for a period of 12 consecutive days. After the 12-day treatment period, the animals were anesthetized with sodium pentobarbital (60 mg/kg body weight, subcutaneously) three hours after the final dose of Neurokinin B, and then sacrificed for further analysis.

Collection of Blood samples

To determine the effect of Neurokinin B treatment on coagulation parameters, the blood was collected directly from left ventricle. The collected blood samples were then transferred into two types of vacutainers; blue top vacutainers – containing 3.2% trisodium citrate (IMPROVE, China) and purple top vacutainers – EDTA.K3 (IMPROVE, China). The blood sample from the blue top vacutainer was used to analyze prothrombin time (PT) and activated partial thromboplastin time (APTT). Plasma was obtained through centrifugation and assayed for PT and APTT using commercially available kits according to manufacturer's instructions. While the blood sample from the purple top vacutainer was used for the analysis of complete blood counts.

Hematological Analysis

The blood sample collected in vacutaner having purple top was promptly utilized for complete blood count analysis. This analysis was performed using an automated hematology analyzer (RT-7600, China). Blood counts provided the estimation of total leukocyte count (103/µl) and differential leukocyte count, red blood cell counts (106/µl), hemoglobin [Hb] (g/dl), mean corpuscular volume [MCV] (fl), mean corpuscular hemoglobin [MCH] (ρg), mean corpuscular hemoglobin concentration [MCHC] (g/dl), hematocrit (%), red blood cell distribution width (RDW), platelet count (103/µl) and mean platelet volume [MPV] (fl).

Analysis of Coagulation Parameters

The bleeding time (BT) in the rabbits was estimated using the Duke's method as per modifications given by Wenche Jy, et al.,.[14] Briefly, a 6mm wide incision was made on the rabbit's ear in the area having no visible vessels with scalpel blade. Timing of the test started with the incision. A filter paper was gently applied to the incision every 30seconds. BT was measured as the duration until bleeding stopped and the disappearance of the blood stains on the filter paper. BT was recoded in seconds.Capillary tube method was used for the estimation of the clotting time (CT). Marginal (lateral) ear vein was used for the collection of blood in capillary tubes. At regular intervals of 30 seconds the capillary tube was broken. Clotting time was measured as the duration until the clot start forming and is measured in second. Estimation of the PT was performed using commercially available Soluplastin kit obtained from the Wiener Lab, Rosario – Argentina. The reagent contains lyophilized rabbit brain thromboplastin with calcium chloride in concentration of 10mM. Reagent was reconstituted by adding 2ml of deionized water in the vial and left for 30 minutes at room temperature with occasional gentle swirling. The kit works on the principle of assessing the time taken for the fibrin clot to form via the extrinsic coagulation pathway upon the addition of calcium thromboplastin to citrated plasma. Citrated plasma and the constituted thromboplastin reagent was preheated at 37°C for 15 minutes in a water bath. Plasma (100 µl) was mixed with thromboplastin reagent (200 µl) in a glass tube. Time till the clot formation was recorded as PT in seconds. Estimation of the APTT was performed using commercially available APTT test kit obtained from the Wiener Lab, Rosario – Argentina. The reagent contains lyophilized rabbit brain

cephalin with ellagic acid and calcium chloride solution (0.025 mol/l). APTT evaluates intrinsic coagulation pathway. The kit works on the principle of assessing the time taken for the coagulation of acitrated plasma via the intrinsic coagulation pathway in the presence of calcium chloride solution and cephalin. As per manufacturer's instructions the citrated plasma, the reagent and the calcium chloride solution was preheated at 37°C for 15 minutes in a water bath. Reagent (100 µl) was mixed with the citrated plasma (100 µl) in a glass tube, swirled gently and incubated at 37°C. Then calcium chloride solution (100 µl) was mixed. Time till the clot formation was recorded as APTT in seconds.

International Normalized Ratio (INR)

Below mentioned formula was used for the calculation of INR in the present study: $INR = (PT/(MNPT))^{ISI}$

Where; PT is prothrombin time of the peptide-treated animals, MNPT is the mean prothrombin time of the distilled water treated control animals and ISI (international-sensitivity-index) for the reagents used, which was 1.1.

Statistical Analysis Data were presented in form of tables and figures as Mean±SEM. SPSS version 26 was used for the analysis of results. Statistical significance was assessed using one way ANOVA which was followed by the post-hoc Tukey's test, with $P<0.05$ was considered statistically significant.

Results

General Observations

Throughout the experiment, all peptide-treated rabbits remained healthy as assessed by their vital signs, overall condition of the body, eating habits, drinking habits, and activity inside the cage. However, a notable exception was observed in the rabbits treated with Neurokinin B that their incised wounds continued to bleed for a much longer duration post-treatment. In contrast, rabbits that served as control and received distilled water treatment showed no excessive bleeding tendency.

Hematological Parameters

The study found a significant increase in the total leukocyte count in the Neurokinin B-treated groups compared to the control group (Table I).

The red blood cell count (RBC) did not show significant differences among the treated and control groups. Parameters such as RDW, hemoglobin levels, MCH, MCHC, MCV and hematocrit did not exhibit significant variations among the groups (Table I).

The platelet count showed a significant decrease in the Neurokinin B-treated groups compared to the control group. While MPV exhibited a significant increase in both Neurokinin B-treated groups compared to the control group (Table I).

Coagulation Parameters

PT and APTT showed significant prolongation in the Neurokinin B-treated groups compared to the control group (Figure 1).PT and APTT showed significant prolongation in the Neurokinin B-treated groups compared to the control group (Figure 2).

International Normalized Ratio (INR)

INR exhibited significant ($P<0.001$) elevation in the Neurokinin B µg group (2.0±0.13) compared to the control group (1.1±0.07), while there is no significant increase in Neurokinin B ηg group (1.32±0.04).

Table 1: Hematological Parameters of the Control and Neurokinin B-Treated Groups

Variables	Control	NeuroKininB ηg µg	NeuroKininB µg
Total Leukocyte Count (10^3/µl)	8.22±1.16	14.88±1.56***	15.73±1.38***
Hemoglobin (g/dl)	10.75±1.76	9.77±0.61	10.15±0.73
Mean Corpuscular Hemoglobin [MCH] (pg)	20.35±0.67	22.20±0.88*	22.27±1.42*
Mean Corpuscular Hemoglobin Concentration [MCHC] (g/dl)	33.84±0.51	35.15±1.09	35.31±2.19
RBC Count	5.29±0.93	4.39±0.22	4.58±.43
Mean Corpuscular Volume [MCV] (fl)	60.10±2.43	63.22±2.15	63.05±2.32
Hematocrit (%)	31.80±5.34	27.78±1.20	28.82±1.98
Red Blood Cell Distribution Width [RDW] (%)	12.10±0.45	12.0±1.16	11.98±0.66
Platelet Count (103/µl)	246.34±28.73	179.67±28.09***	143.50±13.60***
Mean Platelet Volume [MPV] (fl)	6.05±0.27	6.55±0.36*	6.57±0.30*

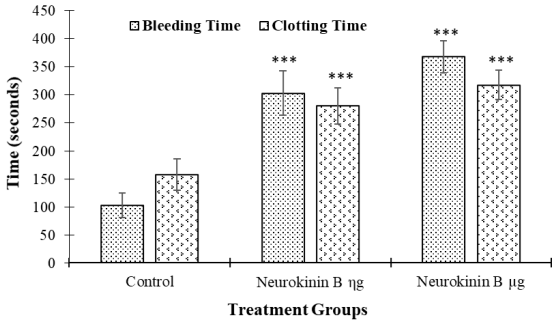

Figure 1: Bleeding time (in seconds) and clotting time (in seconds) of the control and Neurokinin B treated groups (***$P<0.001$ compared to control).

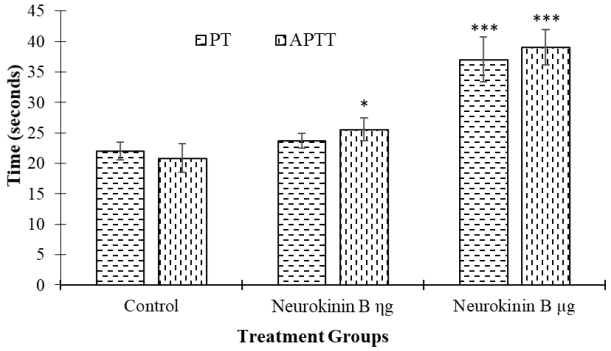

Figure 2: Prothrombin time (in seconds) and activated partial thromboplastin time (in seconds) of the control and Neurokinin B treated groups (***$P<0.001$ and *$P<0.05$ compared to control).

Discussion

The present study investigates the anticoagulation effects of subcutaneously administrated Neurokinin B in adult male rabbits. We observed a dose-dependent impact on the hematological and coagulation parameters following the administration of variable doses of Neurokinin B. We observed a significantly increased bleeding and clotting time with increased PT, APTT, INR and TLC and decreased platelet count in Neurokinin B treated rabbits as compared to control. This effect was dose dependent which potentiated as the dose of Neurokinin B increases (Table I and Figure 2).

Prolonged bleeding time is linked with the thrombocytopenia, impaired platelet ability to adhere with vascular endothelium, von Willebrand disease and disseminated intravascular coagulation. Increased clotting time is associated with hemophilia, deficiencies of the factors involved in clotting pathways (II, V, XI, XII) and congenital coagulation disorders. Elevated PT reflects the activity of the extrinsic coagulation pathway, and is associated with the deficiency of vitamin K or the acute/chronic liver disease. Conversely, APTT evaluates intrinsic coagulation pathway and it is typically performed when there is unexplained bleeding or clotting.[15, 16]

In present study, we observed significantly increased clotting and bleeding times, along with prolonged PT and APTT, suggesting an impact on both intrinsic and extrinsic coagulation pathways. We also noted a marked decrease in platelet count, indicating a disruption in the normal coagulation process. It's worth mentioning that thrombocytopenia is often associated with prolongedBT.13,15In spite of the prolonged BT, PT and APTT, coupled with reduced platelet count, the occurrence of disseminated intravascular coagulation (DIC) as a reason for increased bleeding propensity is highly unlikely.[15] This is supported by the fact that the Neurokinin B treated rabbits remained active, healthy and survived throughout the study period without displaying any signs of internal hemorrhage, sepsis, infection, or liver damage (data not shown). Therefore, our study suggests that Neurokinin B likely blocks the coagulation pathway at its initial stages. Prolongation of the PT and APTT was observed in Neurokinin B treated rabbits which suggests interference with the availability of the vitamin K, however it's important to note that vitamin K deficiency typically does not affect bleeding time or platelet count.[15, 16] Therefore, while it is speculative at this stage to conclude that Neurokinin B directly impedes the availability of vitamin K to stimulate its anticoagulation effects. However, the possible role Neurokinin B in down-regulating the vitamin K dependent pathways, cannot be entirely overruled.[14,15] Neurokinin B has a short plasma half-life and blood samples were collected at least three hours after the last dose of the peptide and anticoagulation profile was assayed. This suggests that the primary anticoagulation effects might occur shortly after peptide administration. This rapid onset of action is similar to anticoagulants like Argatroban and Heparin, which also induce anticoagulation effects in a short time frame.[17] Despite using citrated blood for the conduction of PT and APTT tests—standard for all coagulation tests—the observed anticoagulation effect is more likely attributable to Neurokinin B treatment rather than sodium-citrate.[18] The results of the present study demonstrated a negative association between the thrombocytes and MPV. Presence of bleeding disorders or iron deficiency or anticoagulants may present with a similar scenario.[19] Patients having heterozygous thalassemia may have increased MPV. Conversely a decreased MPV has been observed in subjects undergoing chemotherapy. This converse relationship between thrombocyte size and count is valuable in assessing abnormal thrombocyte production.[17,19] In present study, marked thrombocytopenia was evident with Neurokinin B treatment and a dose dependent effect was noticed with the higher dose accounting for the more severe Neurokinin B-induced thrombocytopenia.[13] Heparin-induced thrombocytopenia is a serious and often underestimated adverse effect of heparin treatment which occurs in about 2% of individuals when they are exposed to heparin for more than four days.[20] Heparin is commonly prescribed for the treatment of deep vein thrombosis and pulmonary embolism. It is a powerful anticoagulant and it significantly increases the formation of thrombin-antithrombin.[15] Although Neurokinin B appears to act similarly to heparin, it is a small peptide, and its mechanisms may differ. The exact mechanism behind Neurokinin B-induced thrombocytopenia remains unknown, however the present data suggested a direct action of the peptide on platelets. Discerning reduction in thrombocytes with increased WBC counts, supports the notion of Neurokinin B-induced thrombocytopenia. Additionally, there was no significant change in hemoglobin content and hematocrit, while mean corpuscular hemoglobin (MCH) increased with Neurokinin B treatment (Table I).

In vivo, Neurokinin B-induced thrombocytopenia might result from modulation of megakaryocyte production. Thrombocyte count can be used as a prognostic index as the production of the thrombocytes was stimulated by many neoplasms through initiation of megakaryocyte production.[21] Although platelet aggregation tests were not conducted in this study, Neurokinin B-induced disaggregation of thrombocytes might be accountable for the observed anticoagulation effects. With Neurokinin B treatment, we observed a significant reduced thrombocyte count with prolonged anticoagulation profiles. These conditions are typically associated with end-stage liver disease, disseminated intravascular coagulation or massive transfusion reactions.[15,16] Although liver function tests were not performed, necropsy of the Neurokinin B-treated rabbits showed no signs of liver damage. Additionally, the animals enrolled in the present study were healthy throughout the experiments and did not show any signs of sepsis as mentioned earlier.

Given that Neurokinin B is a non-toxic peptide, it is likely that the observed anticoagulation effect was attributable to Neurokinin B.

Neurokinin B may exert its effects by down-regulating thromboplastin, thereby deactivating the factors especially VII, X and IX. Another probability is that Neurokinin B directly inhibits thrombin, which can subsequently suppress the subsequent events in the coagulation pathways.[15] Anticoagulatory activity of the Neurokinin B may be activated directly by inhibiting the formation or actions of the thrombin or tissue plasminogen activator. Our results suggests that Neurokinin B can be used as an effective thrombolytic therapy. This potential effect or Neurokinin B warrants additional investigation. It could be highly beneficial for treating venous thromboembolism. Current treatment options for thromboembolism include unfractionated and low molecular weight heparins (LMWHs), warfarin and inferior vena cava filters.[22]

Limited data is available on the effect of tachykinins on the hematological and coagulation profile. It has been demonstrated that SP induces platelet aggregation and degranulation. This effect of SP has been supposed to be mediated through tachykinin receptors, specifically NK1 (the receptor for SP) and NK3 (the receptor for neurokinin B).[8] Another study highlighted the role of tachykinins (SP and Endokinins A and B) in the activation of platelet function and the formation of thrombi.[23] Furthermore, additional research indicated that the pro-thrombotic effects of tachykinins on platelets are mediated via the neurokinin 1 receptor.[12] In another study, it was found that Substance P and Neurokinin A are crucial for neutrophil priming and the release of superoxide anions from neutrophils.[24]

Anticoagulants often use GPCR to mediate their anticoagulant effects and aggregation of platelets. [15,16] Notably, Neurokinin B also exerts its actions through a GPCR.1 Therefore, it is plausible that Neurokinin B promotes its anticoagulation effects by using intracellular second messenger pathways.

To our knowledge, this study is the first to explore the effects of continuous subcutaneous administration of varying doses of Neurokinin B on hematological and coagulation parameters. As a result, there are currently no other studies available for direct comparison.

Limitations

The study observed significant changes in platelet count but did not analyzed platelet aggregation for understanding the mechanisms behind Neurokinin B-induced thrombocytopenia. Additionally, liver function tests were not performed, though necropsy showed no signs of liver damage. The focus was limited to specific hematological and coagulation parameters, and including additional parameters like fibrinogen levels and D-dimer could provide a more comprehensive understanding. The present study is preliminary and warrants detailed biochemical investigation into the anticoagulation effects of Neurokinin B, as the exact mechanisms behind the observed effects remain unclear and require further exploration of the molecular pathways involved for its potential use as a therapeutic agent.

Conclusion

Neurokinin B administration led to remarkable results, with increase in leukocyte count, however at the same time it altered the platelet dynamics with decreased count and increased volume, and also prolonged BT, CT, PT and APTT. Further research is needed to understand these effects and their clinical significance.

To conclude, Neurokinin B significantly impacts blood and coagulation parameters, and understanding these effects may provide new therapeutic insights for managing prevalent blood disorders.

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