

Original Research Article

PROTECTIVE EFFECTS OF SELECTED ANTIOXIDANTS ON THE LEAD-INDUCED THROMBOCYTOPENIA IN MALE WISTAR RAT

Abstract

Aim: The study was undertaken to investigate the protective effects of selected antioxidants on lead-induced thrombocytopenia, using male Wistar rats as experimental models.

Methodology: One hundred and sixty-two male Wistar rats with weight between 180 and 200 g were obtained from the Experimental Animal Farm of the University of Port Harcourt, Nigeria. The Wistar rats were housed in animal wooden cages in a well-ventilated experimental room and allowed to acclimatize for a period of two weeks before the commencement of treatments. The control group (group 1) was orally given 0.5ml of distilled water while the treatment groups (groups 2 to 9) were orally given different substances as follows: 10mg/Kg Body Weight (BW) of Lead only, 200mg/Kg BW of Vitamin C only, 1000iu/Kg BW of Vitamin E only, 10mg/Kg BW of Lead + 200mg/Kg BW of Vitamin C, 10mg/Kg BW of Lead + 1000iu/Kg BW of Vitamin E, 10mg/Kg BW of Lead + 40mg/BW Levamisole, 10mg/Kg BW of Lead + 200mg/Kg BW of Vitamin C + 40mg/BW Levamisole and 10mg/Kg BW of Lead + 1000iu/Kg BW of Vitamin E + 40mg/Body Weight Levamisole respectively once a day. The experiment was conducted in three phases (phases 1 to 3), which lasted for 7 days (acute phase), 30 days (sub-acute phase) and 60 days (chronic phase). At the expiration of the experimentation for each phase, five (5) rats were sacrificed blood samples were collected from each rat and examined for thrombocyte parameters. The effects of treatment with lead and antioxidants were compared with the control group.

Result: There was a significant decrease in the concentrations of Plateletcrit (PCT) and Platelet count (PLT) and a significant decrease in Mean platelet volume (MPV), Platelet distribution width (PDW), Platelet large cell ratio (PLCR) and Platelet large cell count (PLCC) all in group 2, with respect to the control. There was also a significant increase in PLT in the lead group with respect to the control, in all the phases. Also, there was a significant increase in the concentrations of PCT (in groups 5, 6, 8 and 9) and PLT (in groups 5, 8, 9), all with respect to the lead group. Finally, there was a significant decrease in the concentrations of MPV (in groups 5 to 9), PDW (in groups 5 to 9), PLCR (in group 5 to 8) and PLCC (in group 9), all with respect to the lead group.

Conclusion: The antioxidants have therefore demonstrated the ability to ameliorate the lead-induced Toxicity on the platelet parameters.

Keywords: *Lead, thrombocytes, Antioxidants, Ameliorate and Toxicity.*

INTRODUCTION

Lead toxicity remains a global health concern due to its pervasive environmental presence and harmful effects on various biological systems. Chronic exposure to lead is associated with hematological abnormalities, including thrombocytopenia, a condition characterized by a decrease in platelet count and altered platelet indices, which can result in impaired hemostasis and increased bleeding risks. ^[1] The underlying mechanism of lead-induced thrombocytopenia is primarily attributed to oxidative stress, which

disrupts hematopoietic processes by generating reactive oxygen species (ROS) and depleting antioxidant defenses. [2]

Oxidative stress plays a pivotal role in lead-induced cytotoxicity, emphasizing the need for therapeutic strategies that can mitigate its effects. Antioxidants, with their ability to neutralize ROS and enhance the body's natural antioxidant capacity, have been proposed as potential therapeutic agents. Studies have shown that both natural and synthetic antioxidants can alleviate oxidative damage in lead-exposed models by restoring redox balance and protecting cellular components, including those in the hematopoietic system. [3]

Antioxidants have emerged as a promising therapeutic approach to mitigate the adverse effects of lead-induced oxidative stress. Lead toxicity induces the generation of reactive oxygen species (ROS), which disrupt cellular homeostasis and contribute to immune system dysfunction. [4] Antioxidants, such as vitamin C, vitamin E, and selenium, counteract ROS, restoring cellular function and reducing oxidative damage. [1] Studies have highlighted the potential of antioxidants to protect immunoglobulins from lead-induced toxicity, preserving immune competence and reducing systemic inflammation. [5,6]

This study aims to investigate the ameliorative potential of antioxidants on lead-induced thrombocytopenia in male Wistar rats. By examining the effects of antioxidants on platelet count and indices, the research seeks to elucidate their protective mechanisms and provide insights into their therapeutic relevance in managing lead-induced haematotoxicity.

MATERIALS AND METHOD

One hundred and sixty-two (162) male Wistar rats with weight between 180 and 200 g were obtained from the Experimental Animal Farm at the University of Port Harcourt, Nigeria. The Wistar rats were housed in animal wooden cages in a well-ventilated experimental room. The rats were allowed to acclimatize for a period of two weeks before the commencement of treatments. The rats had free access to standard rat chow and clean water ad libitum. Handling of animals was in accordance with relevant institutional and ethical guidelines as approved for scientific study. After acclimatization, the rats were divided into nine (9) groups. The control group (group 1) was orally given 0.5ml of distilled water only, group 2 was given 10mg/Kg Body weight of Lead only, group 3 was given 200mg/Kg Body Weight of Vitamin C only, group 4 was given 1000iu/Kg Body Weight of Vitamin E only, group 5 was given 10mg/Kg Body Weight of Lead + 200mg/Kg Body Weight of Vitamin C, group 6 was given 10mg/Kg Body Weight of Lead + 1000iu/Kg body Weight of Vitamin E, group 7 was given 10mg/Kg Body Weight of Lead + 40mg/Body Weight Levamisole, group 8 was given 10mg/Kg Body Weight of Lead + 200mg/Kg Body Weight of Vitamin C + 40mg/Body Weight Levamisole while group 9 was given 10mg/Kg Body Weight of Lead + 1000iu/Kg Body Weight of Vitamin E + 40mg/Body Weight Levamisole. These administrations were done once a day. The experiment was conducted in three phases (phases 1 to 3). Phase 1 (acute phase) lasted for 7 days, phase 2 (sub-acute phase) lasted for 30 days while phase 3 (chronic phase) lasted for 60 days. At the expiration of the experimentation for each phase, five (5) rats were sacrificed from each group and blood samples were collected from each rat in an EDTA bottle. The blood samples were collected, using the method of cardiac

puncture, after each rat has been anaesthetized in a desiccator, using diethyl ether and examined for thrombocyte parameters (PCT, PLT, MPV, PDW, PLCR and PLCC). The effects of treatment with lead and antioxidants were compared with the control group.

The results were subjected to statistical analysis using statistical package for social sciences (SPSS) version 20.0. Data are presented as mean \pm SEM. Difference of means were considered significant at P value less than 0.05.

RESULTS

Table 1: Ameliorating effects of synthetic antioxidants on lead-induced toxicity on the concentration of Thrombocytes after 7 days

Thrombocyte parameters	Grp 1	Grp 2	Grp 3	Grp 4	Grp 5	Grp 6	Grp 7	Grp 8	Grp 9
Plateletocrit (PCT)	0.58 ± 0.03	0.39 ^a ± 0.11	0.67 ± 0.05	0.65 ± 0.0	0.57 ^b ± 0.06	0.55 ^b ± 0.05	0.49 ± 0.03	0.61 ^b \pm 0.05	0.70 ^b \pm 0.05
Platelet count (PLT)	286.0 ± 14.9 5	160.0 ^a ± 16.05	338.0 ± 18.1 4	327.50 ± 20.97	274.00 ^b ± 21.88	190.0 ± 22.5 3	175.00 ± 19.25	270.00 ^b ± 20.13	275.00 ^b ± 21.04
Mean Platelet volume (MPV)	11.20 ± 2.82	23.44 ^a ± 1.68	8.62 ± 2.38	11.10 ± 2.78	10.16 ± 1.19	8.12 ± 1.07	9.98 ^b ± 0.61	8.28 ± 0.66	13.24 ^b ± 1.85
Platelet distribution width (PDW)	11.20 ± 2.82	23.44 ^a ± 1.68	8.62 \pm 2.38	9.40 ± 2.57	10.7 ^b ± 0.97	7.82 ^b ± 0.98	9.68 ^b ± 0.82	10.16 ^b \pm 1.67	12.45 ^b ± 2.17
(Platelet large cell Ratio (PLCR)	4.60 ± 1.29	5.74 ^a ± 0.96	3.16 ± 0.28	5.20 ± 1.18	3.62 ^b ± 0.12	4.46 ^b ± 0.18	3.90 ^b ± 0.23	5.40 ^b ± 0.97	5.02 ± 0.86
(Platelet large cell count) PLCC	3.68 ± 0.50	6.82 ^a \pm 1.10	3.42 ± 0.31	5.68 ± 1.39	3.34 ± 0.16	4.12 ± 0.22	4.32 ± 0.16	4.26 ± 0.70	5.84 ^b ± 1.06

^a and ^b denote significant differences when compared with the control and lead groups respectively, at $p < 0.05$.

The effects of the administration of antioxidants (Vitamin C, Vitamin E and Levamisole) on lead-induced toxicity on the concentration of thrombocytes (PCT, PLT, MPV, PDW, PLCR and PLCC) after 7 days are presented in table 1 above.

There was a significant decrease in the concentration of PCT and PLT and a significant increase in the concentrations of MPV, PDW, PLCR and PLCC, all in group 2, with respect to the control group. Also, there was a significant increase in the concentrations of PCT (in groups 5, 6, 8 and 9) and PLT (in groups 5, 8, 9), all with respect to the lead group. Finally, there was a significant decrease in the concentrations of MPV (in groups 5 to 9), PDW (in groups 5 to 9), PLCR (in group 5 to 8) and PLCC (in group 9), all with respect to the lead group.

Table 2: Ameliorating effects of synthetic antioxidants on lead-induced toxicity on the concentration of Thrombocytes after 30 days

Thrombocyte parameters	Grp 1	Grp 2	Grp 3	Grp 4	Grp 5	Grp 6	Grp 7	Grp 8	Grp 9
Plateletocrit (PCT)	0.60 ±0.03	0.51 ^a ±0.02	0.66 ^a ±0.02	0.60 ± 0.15	0.62 ^b ±0.02	0.61 ^b ±0.01	0.62 ^b ±0.01	0.59 ^b ± 0.01	0.62 ^b ±0.02
Platelet count (PLT)	288.80 ±14.86	101.0 ^a ±4.58	313.00 ±24.47	316.00 ±17.65	229.0 ^b ±53.51	148.0 ±8.31	196.0 ^b ±24.97	162.00 ±3.00	301.0 ^b ± 26.85
Mean Platelet volume (MPV)	10.96 ±2.70	24.16 ^a ±3.01	6.70 ±0.64	8.04 ±0.25	7.36 ^b ±0.19	7.40 ^b ± 0.40	7.62 ±0.38	5.86 ±1.11	12.76 ^b ±2.35
Platelet distribution width (PDW)	4.72 ±1.40	16.18 ^a ±1.97	3.82 ±1.27	4.08 ±1.47	5.72 ^b ± 1.34	3.72 ^b ±0.20	6.16 ^b ± 1.25	8.76 ^b ±0.15	9.84 ^b ±0.55
(Platelet large cell Ratio (PLCR)	3.46 ±0.67	5.96 ^a ±0.89	2.92± 0.53	2.62 ±0.50	4.96± 0.84	5.16 ±0.84	5.54 ±0.81	5.44 ±0.90	5.22 ±0.86
(Platelet large cell count) PLCC	50.80 ±5.79	64.60 ±9.09	42.00 ±5.41	33.20 ±4.20	46.40 ±6.52	56.80 ±6.52	59.80 ±11.72	43.40± 9.53	56.60 ±9.40

^a and ^b denote significant differences when compared with the control and lead groups respectively, at p<0.05.

The effects of the administration of antioxidants (Vitamin C, Vitamin E and Levamisole) on lead-induced toxicity on the concentration of thrombocytes (PCT, PLT, MPV, PDW, PLCR and PLCC) after 30 days are presented in table 2 above.

There was significant decrease in the concentration of PCT and PLT and a significant increase in the concentrations of MPV, PDW and PLCR, all in then lead group, with respect to the control group. Also, there was a significant increase in the concentrations of PCT in groups 3, with respect to the control group. Again, there was a significant increase in the concentration of PCT (in groups 5 to 9) and PLT (in groups 5, 7, 8 and 9), with respect to the lead group. Finally, there was a significant decrease in the concentrations of MPV (in groups 5 to 9) and PDW (in groups 5 to 9), all with respect to the lead group.

Table 3: Ameliorating effects of synthetic antioxidants on lead-induced toxicity on the concentration of Thrombocytes after 60 days

Thrombocyte parameters	Grp 1	Grp 2	Grp 3	Grp 4	Grp 5	Grp 6	Grp 7	Grp 8	Grp 9
Plateletocrit (PCT)	0.66 ±0.03	0.54 ^a ± 0.04	0.75 ^a ±0.04	0.75 ^a ±0.03	0.59 ±0.01	0.60 ±0.01	0.60 ±0.01	0.63 ^b ±0.01	0.58 ±0.02

Platelet count (PLT)	294.40 ±14.45	148.0 ^a ±9.70	347.00 ±23.54	314.00 ±15.92	223.0 ^b ±30.48	244.0 ^b ±10.17	241.8 ^b ±17.00	268.0 ^b ±30.52	236.0 ^b ±26.38
Mean Platelet volume (MPV)	10.62 ±2.72	12.70 ±2.16	8.98 ±0.74	8.18 ±2.23	9.68 ±1.33	13.38 ±0.79	16.86 ±0.41	9.92 ±1.15	6.76 ^b ±0.11
Platelet distribution width (PDW)	4.56 ±1.43	6.70 ±2.06	4.50 ±0.36	3.52 ±1.26	4.88 ±0.66	4.86 ±1.59	5.56 ±1.97	5.16 ±1.61	5.92 ±2.02
Platelet large cell Ratio (PLCR)	3.34± 0.60	22.28 ^a ± 9.46	2.74 ±0.49	3.04 ±0.53	12.48 ^b ±0.83	8.06 ^b ±1.41	8.74 ^b ±1.33	14.22 ±1.96	9.88 ^b ±1.50
(Platelet large cell count) PLCC	49.00 ±5.20	57.90 ±3.65	13.80 ^a ±2.31	42.00 ±7.52	20.0 ^b ±4.29	50.00 ±2.57	40.80 ^b ±3.36	51.00 ±2.51	47.80 ±3.25

^a and ^b denote significant differences when compared with the control and lead groups respectively, at $p < 0.05$

The effects of the administration of antioxidants (Vitamin C, Vitamin E and Levamisole) on lead-induced toxicity on the concentration of thrombocytes (PCT, PLT, MPV, PDW, PLCR and PLCC) after 60 days are presented in table 3 above.

There was significant decrease in the concentration of PCT and PLT and a significant increase in the concentrations of PLCR, all in group 2, with respect to the control group. There was also a significant increase in the concentration of PCT (in groups 3 and 4) and a significant decrease in PLCC in group 3, all with respect to the control. Again, there was a significant increase in the concentration of PCT (in group 8) and PLT (in groups 5 to 9), all with respect to the lead group. Finally, there was a significant decrease in the concentrations of MPV (in group 9), PLCR (in group 5, 6, 7 and 9) and PLCC (in groups 5 and 7), all with respect to the lead group.

DISCUSSION

The findings of this study demonstrate the protective effects of antioxidants on lead-induced thrombocytopenia (PCT, PLT, MPV, PDW, PLCR and PLCC) disorders, with notable variations in response observed across different time intervals (7days, 30days, and 60 days). These results underscore the therapeutic potential of antioxidants such as Vitamin C, Vitamin E, and Levamisole in mitigating the immunosuppressive effects of lead toxicity.

Platelet count (PLT), plateletcrit (PCT), mean platelet volume (MPV), platelet distribution width (PDW), platelet large cell ratio (PLCR), and platelet large cell count (PLCC), are key platelet indices which are measured routinely as part of a complete blood count (CBC) and are essential for understanding hemostasis, thrombopoiesis, and associated pathologies. [7]

Platelet count (PLT) refers to the number of platelets per microliter of blood, typically ranging from 150,000 to 450,000/ μ L in healthy individuals. Platelets are produced from megakaryocytes in the bone marrow and play a central role in hemostasis by forming platelet plugs and contributing to clotting cascades. [8] A low PLT (thrombocytopenia) can result from conditions such as bone marrow failure, autoimmune diseases, or infections, leading to an increased risk of bleeding. Conversely, high PLT (thrombocytosis) is often associated with myeloproliferative disorders or inflammation, increasing the risk of thrombosis. [9]

Plateletcrit (PCT) measures the total platelet mass in the blood, expressed as a percentage of total blood volume. It is calculated as:

$$PCT = PLT \times MPV / 10,000$$

The reference range for PCT is 0.2–0.36%. PCT reflects overall platelet biomass and is a more comprehensive indicator of thrombopoiesis and platelet activation compared to PLT alone. ^[10]

Elevated PCT levels are associated with conditions such as thrombocytopenia, while reduced PCT indicates thrombocytopenia or reduced platelet production. PCT also correlates with cardiovascular risk and disease severity in disorders like sepsis and myocardial infarction. ^[11]

Mean platelet volume (MPV) represents the average size of circulating platelets, with a normal range of 7.5–12 fL. It is an indirect marker of platelet production and activation. Larger platelets, reflected by higher MPV, are metabolically and enzymatically more active, often seen in prothrombotic states (Martin et al., 2010). An increased MPV is associated with conditions like diabetes mellitus, hypertension, and acute coronary syndrome, where platelet activation plays a critical role. Decreased MPV can indicate aplastic anemia or marrow suppression due to chemotherapy. ^[12]

Platelet distribution width (PDW) measures the variation in platelet size, reflecting the heterogeneity of platelet populations. It is expressed as a percentage, with normal values ranging from 15–17%. Higher PDW indicates increased anisocytosis, often linked to activated platelet production and fragmentation of megakaryocytes. ^[13] PDW is elevated in thrombotic disorders, infections, and inflammatory diseases, highlighting the dynamic response of platelets in these conditions. It has been proposed as a marker for the prognosis of diseases like stroke and sepsis. ^[14]

Platelet large cell ratio (PLCR) quantifies the proportion of large platelets (greater than 12 fL) in the circulation, expressed as a percentage. Normal PLCR values range from 15–35%. Higher PLCR is indicative of increased platelet turnover and activation, as seen in conditions like myocardial infarction, preeclampsia, and essential thrombocytopenia. ^[15] An elevated PLCR suggests enhanced megakaryocyte activity or platelet destruction, while low PLCR may point to bone marrow suppression or impaired thrombopoiesis.

Platelet Large Cell Count (PLCC) measures the absolute number of large platelets in circulation, combining aspects of PLT and PLCR. It serves as a marker of thrombopoietic activity and platelet destruction. Elevated PLCC is seen in thrombocytosis and inflammatory states, while decreased PLCC is observed in aplastic anemia or marrow failure. ^[16] PLCC has been used in conjunction with other platelet indices to evaluate cardiovascular diseases and monitor responses to antiplatelet therapy.

Together, these platelet indices provide a comprehensive picture of platelet function, size, and activity. Their clinical applications span haematology, cardiology, and critical care and aiding in the diagnosis and monitoring of various conditions. For instance, combined assessment of MPV and PDW can differentiate between reactive and clonal thrombocytosis, while PCT and PLCC offer insights into platelet mass and function in thrombotic disorders. ^[7]

This research evaluates the modulatory roles of antioxidants (Vitamin C, Vitamin E, and Levamisole) on thrombocyte indices (PCT, PLT, MPV, PDW, PLCR, and PLCC) in lead-induced toxicity in male Wistar rats. Thrombocyte indices reflect platelet production, morphology, and function, which are critical for hemostasis and immune regulation. Lead-induced toxicity disrupts these indices through oxidative stress and inflammatory processes. Below is a phase-wise discussion of the findings and their comparison with similar studies.

Lead exposure (Group 2) after 7 days resulted in significant reductions in PCT and PLT levels, indicating thrombocytopenia. Conversely, MPV, PDW, PLCR, and PLCC were significantly elevated. These changes suggest that lead induces platelet destruction and compensatory mechanisms, such as the release of immature, larger platelets with higher MPV and PDW values. This is consistent with the findings of others who reported that lead-induced oxidative stress damages platelet membranes and promotes inflammation, leading to altered platelet dynamics. ^[17]

Antioxidant administration effectively ameliorated the lead-induced changes. Groups 5, 6, 8, and 9 showed significant increases in PCT and PLT, while MPV, PDW, PLCR, and PLCC significantly decreased compared to the lead group. These improvements suggest that antioxidants mitigate oxidative stress, protect platelet integrity, and restore thrombocyte homeostasis. Vitamin C and E, as potent antioxidants, likely scavenged reactive oxygen species (ROS) and stabilized platelet membranes, while Levamisole's immunomodulatory effects enhanced thrombocyte recovery. These observations align with the work of others, who demonstrated that antioxidants restore platelet counts and morphology by reducing oxidative damage in toxicological models. ^[18]

In the sub-acute phase, lead continued to reduce PCT and PLT levels while significantly increasing MPV, PDW, and PLCR compared to the control. The persistent thrombocytopenia reflects chronic oxidative damage to megakaryocytes (platelet precursors) and sustained platelet destruction. ^[19] Elevated MPV and PDW indicate the release of larger, immature platelets in response to ongoing thrombocyte depletion. The antioxidants maintained their protective effects, as evidenced by increased PCT and PLT levels in Groups 5 to 9. These results highlight the restorative role of antioxidants in reversing lead-induced thrombocytopenia over longer durations. Additionally, the significant reductions in MPV and PDW in Groups 5 to 9 indicate improved platelet morphology and reduced compensatory stress on thrombopoiesis. The significant improvement in Groups 8 and 9, which involved combined administration of Vitamin C, Vitamin E, and Levamisole, suggests a synergistic effect. This is in line with studies by other researchers, who reported enhanced efficacy of combined antioxidant therapies in mitigating heavy metal toxicity. ^[20] Lead exposure in the chronic phase (60 days of administration) resulted in consistent reductions in PCT and PLT levels and increased PLCR, indicating sustained thrombocytopenia and altered platelet turnover. Chronic exposure may exacerbate oxidative damage and inflammation, further impairing thrombopoiesis and promoting platelet destruction. The antioxidant administration showed phase-specific benefits. Groups 8 and 9 demonstrated significant improvements in PCT and PLT compared to the lead group, indicating sustained restoration of thrombocyte levels. However, reductions in MPV, PLCR, and PLCC suggest that

antioxidants also enhanced platelet quality and reduced the release of immature platelets. The effectiveness of combined therapies (Vitamin C + Levamisole or Vitamin E + Levamisole) in Group 8 and 9 underscores the benefits of targeting multiple pathways of oxidative stress and inflammation. The ability of Vitamin C and Vitamin E to enhance platelet count and morphology is supported by the results of others, who demonstrated their roles in stabilizing cell membranes and reducing oxidative injury. Levamisole's role in modulating immune and hematopoietic function likely complements the actions of antioxidants. [20]

The results align with a growing body of evidence that supports the protective roles of antioxidants in heavy metal-induced toxicity. For instance, studies by other group of researchers reported that antioxidants like Vitamin C and E ameliorate oxidative stress and enhance platelet recovery in heavy metal toxicity. [2,1] Again, the observed reductions in MPV and PDW with antioxidant therapy are consistent with findings by others, who highlighted the role of antioxidants in normalizing platelet size and function by reducing oxidative and inflammatory stress on megakaryocytes.[18] Furthermore, the combination of Vitamin C, Vitamin E, and Levamisole demonstrated superior efficacy in reversing thrombocyte abnormalities. This finding corroborates other researchers, who emphasized the importance of combination therapies in addressing the multifaceted effects of lead toxicity.[19]

These results therefore demonstrate that lead exposure induces significant alterations in thrombocyte indices, reflecting thrombocytopenia and compensatory stress and that antioxidants, particularly in combination, effectively mitigate these effects by restoring platelet count, morphology, and function. The findings also highlight the therapeutic potential of Vitamin C, Vitamin E, and Levamisole in addressing lead-induced hematological dysfunction and underscore the benefits of multi-antioxidant therapies for synergistic outcomes.

CONCLUSION

The findings of this study underscore the efficacy of antioxidants in ameliorating lead-induced toxicity on thrombocytes, with consistent improvements observed across all the duration of administration. The results suggest that antioxidants such as Vitamin C, Vitamin E, and Levamisole may serve as therapeutic agents to mitigate lead-related thrombocyte dysfunction, with potential implications for managing environmental and occupational lead exposure. Further studies are however recommended to explore the underlying mechanisms and optimize their therapeutic protocols.

ETHICAL APROVAL

All authors hereby declare that "Principles of laboratory animal care" were followed. All experiments have been examined and approved by the appropriate ethics committee.

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