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Research Article

Petroleum contamination altered neuromuscular function and structural integrity in the sciatic nerve-gastrocnemius muscle preparation of toad

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ABSTRACT
Gasoline (petrol) and other petroleum products are in increasing use among the populace. Environmental contamination due to oil exploration, exploitation, distribution and even criminal vandalisation have increased contact and exposure to these products. Indiscriminate abuse of petrol is not limited to automechanics that suck it with the mouth, wash hands and legs with it, it also extends to those who use it as solvents, sniff it for 'pleasure', or use it as insect repellent. We have therefore investigated the effects of gasoline on neuromuscular function. The toad sciatic nerve and gastrocnemius muscle was used. The tissues were placed in a muscle bath containing 50ml of amphibian ringer solution. Graded concentration of gasoline was introduced into the tissue bath for ten minutes. The preparations were stimulated through the nerve and threshold voltage, maximal voltage, latent period, contraction and relaxation profiles were recorded using the Kymograph (Harvard Apparatus). Results from this study show that 0.4% - 2.0% concentration of petrol had significant effect on velocity of shortening, height of contraction and contraction period ($p<0.05$). Lower concentrations increased motor activity with little or no effect on threshold or maximal voltages, absolute refractive period and relaxation period. At 4.0% concentration all neuromuscular activity ceased. Histological investigations revealed tissue damage, disruption of membranes and infiltration and tissue oedema. Therefore it is concluded that petrol may have a toxic effect on neuromuscular structure and function.

Keywords: Gasoline, neuromuscular function, sciatic nerve, gastrocnemius muscle, oedema.

INTRODUCTION

Interest in nervous system toxicology has been growing in recent years, not only because of increased public concern over the impact of toxic agents on human health and the quality of life, but also because the nervous system has been shown to be particularly vulnerable to chemical insult. There has been an increased demand for improved methods for the detection of neurotoxic effects and the assessment of health risks within the field of occupational and environmental health and safety [1;2]. Re-occurring oil and petroleum products spillages is a growing concern in Nigeria. The manner of the effects of these factors include: direct lethal toxicity, sub-lethal disruption of physiological and behavioral activities. This leads to death owing to the interference with feeding and reproduction, direct coating or painting, entry of hydrocarbons into the food web, and alteration of biological habitats and is linked with hepatic encephalopathy and other neurological diseases[3]. Studies of neurotoxicity in model animal systems are essential as neurotoxicity data can be extrapolated to humans.

In this study, the effect of gasoline is tested on the sciatic nerve and gastrocnemius muscle of the toad, *Bufo regularis* in vitro. It is believed that the results of this study will serve as basis for further research on the toxic effect of petroleum products. Altered cholesterol and lipid levels have been implicated in many neurodegenerative disorders [4] including Niemann-pick disease, which is associated with a reduced level of plasma membrane cholesterol[5], changes in cholesterol levels have also been linked to Alzheimer's disease[6].

The human central nervous system is an important target for manganese intoxication which causes neurological symptoms similar to those of Parkinson's disease[7]. The prospect of a worldwide manganese exposure is once again attracting attention as increase in environmental manganese concentration have been recorded relative to traffic density. Abbot[8] reported that experimental and occupational exposure to oxygenate fuel additives in reformulated gasoline induces neurological symptoms. Petrol sniffing is associated with dysfunctions that range from subtle cognitive impairment to encephalopathy and death[9]. Petrol sniffing causes programmed decline of cognitive function that eventually leads to permanent neurological changes. Many substances are known to produce alterations in the peripheral nervous system [1, 10]. Conduction velocity, the speed at which action potentials are conducted along axons and nerves, is the most widely used measure of peripheral nerve function. Many other techniques besides conduction velocity have been applied

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1: Table of values showing the effect of varying concentrations of petrol on contraction and relaxation periods for nerve-muscle preparation.

Petrol Concentration (%) v/v.	Control (0%)	0.4%	0.8%	1.2%	1.6%	2.0%
Mean Contraction Period (milliseconds)	73.0	78.0	68.0	74.0	68.0	67.0
±SEM	3.0	3.0	1.0	2.0	1.0	2.0
Mean Relaxation period (milliseconds)	72.0	74.0	72.0	66.0	66.0	66.0
±SEM	3.0	1.0	5.0	2.0	2.0	1.0

**P-value = 0.0104, P<0.05 (Significant)

in the assessment of peripheral nerve function. They include assessment of the refractory period [11], assessment of the extent to which axons and nerves can follow trains of stimuli occurring at high rates[12], accommodation indices and the use of collision techniques for selectively blocking activity of some nerve axons to study others. Some of these techniques will certainly provide even greater sensitivity than the simple velocity measurements in common use.

Toxic agents that exhibit a preference for the distal ends of long peripheral nerves, a dying-back neuropathy, or distal axonopathy might be expected to alter or impair the sensory function of these receptors[13], motor nerve terminals[14], and primary afferent terminal[15] has been reported to be compromised by toxic agents, long before any alterations are detectable in conduction parameters. Another advantage of these techniques is that, not only can the presence of neurotoxicity be detected, but the site(s) of neurotoxic action can also be investigated.

The complexity of motor activity is emphasized by the finding that low-level exposure to volatile organic solvents increases activity, whereas high level exposure decreases it [16]. Positive results in a motor activity test usually require further testing to identify the precise function affected. Due to its inherently greater resolution as compared to light microscopy, electron microscopy may be used to identify the subcellular or organellar target(s) for a neurotoxic chemical[17,18]. In testing of chemicals for neurotoxicity, electron microscopy studies should be confined to those studies where there is a specific need to better characterize and more clearly define ultrastructural effects. Due to the small tissue sample size used for electron microscopy, proper tissue selection is absolutely essential to ensure that changes which are observed by light microscopy are selected for ultrastructural examination. Electron microscopy is a technique which is to be used in addition to light microscopy to elucidate changes in tissue ultrastructure.

MATERIALS AND METHODS

Toad sciatic nerve gastrocnemius muscle preparation was used for these studies (n = 5 in each group). Toads were supplied by the Department of Laboratory Animal Research and kept in a cool and moist environment in the Amphibian room of the Laboratory Animal Centre until required. The toads were pithed at the foramen magnum and the pithing needle was advanced towards the brain to destroy the brain so as to abolish pain and movement. The skin was cut round in the middle and then pulled off to reveal the leg muscle and lower back. The left and right sciatic nerves were isolated and freed. The nerves were removed with a portion of the vertebra to prevent damage to nerve cell bodies in the anterior grey column. The exposed tissue was moistened to prevent desiccation and the excised tissue placed in Petri dish of amphibian Ringer solution.

Physiological Studies: The amphibian Ringer was prepared using the method described by Andrew[19] (1969). The compositions of the physiological solution include: Sodium Chloride, NaCl 6.5g, Potassium Chloride KCl 0.14g, Calcium Chloride $CaCl_2$ 0.12g, Sodium Hydrogen Carbonate $NaHCO_3$ 0.20g, Sodium Hydrogen Phosphate NaH_2PO_3 0.01g, Glucose $C_6H_{12}O_6$ 2.0g in 1 litre of Distilled Water H_20.

The gastrocnemius muscle was mounted in a muscle bath containing known volume of the amphibian ringer solution. A thread was tied to the Achilles tendon and the other end tied to the effort arm of the kymograph (Harvard Apparatus). The sciatic nerve was placed on the stimulating electrodes which are connected to the stimulator of the kymograph. The threshold voltage was determined by starting at very low voltage, then increasing the voltage in 0.05volts (width 0.5ms) steps while applying a single stimulus between increases, until the first recordable twitch was obtained. The maximal voltage was also determined as the voltage that gave the highest contraction beyond which there was no further increase. Two times the maximal voltage was used for subsequent tests.

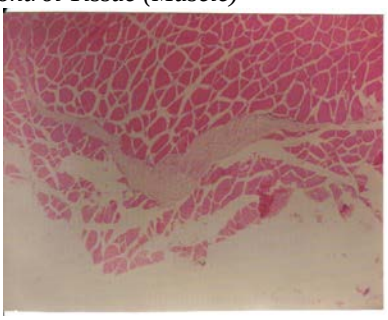
Following the determination of maximal voltage, the Kymograph drum was set at the highest speed (625mm per sec). The drum contact marker is made to trigger a single maximal twitch. The kymograph gear is turned to neutral and rotated by hand to mark the point where the stimulus was applied on the nerve [20]. By separating the two points of the contact marker, two separate stimuli were applied to the nerve at various intervals to determine the absolute refractive period [11, 21].

This setup was used to determine, latent period, contraction and relaxation times, absolute refractory period and heights of contraction. Velocity of shortening was computed from displacement and time of contraction. Various concentrations of gasoline in Ringer 0.4% - 2.0%, were introduced into the tissue bath (50ml) similar to the method used by Anigbogu,[22].

Histopathological Studies: The tissues exposed to various concentrations of gasoline in Ringer used were preserved /fixed in formalin (10%) after recordings were taken. After fixation for more than 24 hours, the tissues were processed using 24hrs automated tissue processor. The

Plates And Figures- Petrol Contamination And Neuromuscular Function

Control Tissue (Muscle)



X40

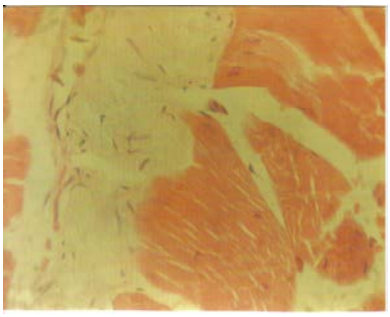


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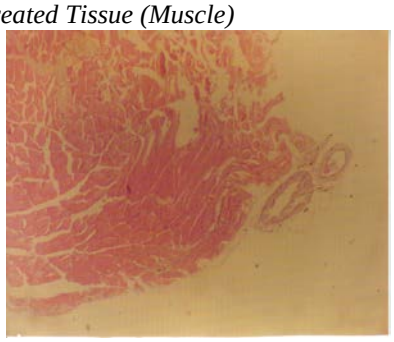
Plate 1. Photomicrograph of the cross section of the gast rocnemius muscle of the toad (Control, 0% petrol) (H&E stain).

Treated Tissue (Muscle)



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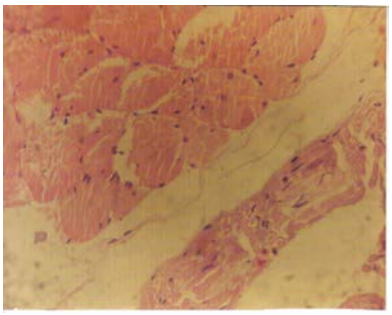
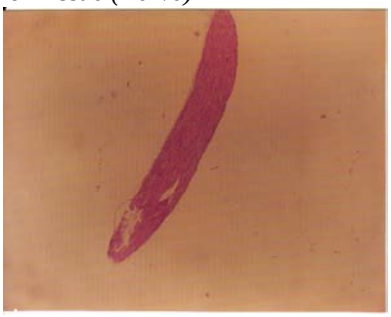


X400

Plate 2. Photomicrograph of the cross section of the gastrocnemius muscle of the toad (2.0% petrol) (H&E stain).

Isolated tissue –nerves: plates 3 and 4

Control Tissue (Nerve)



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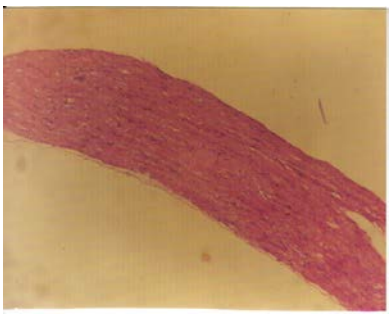
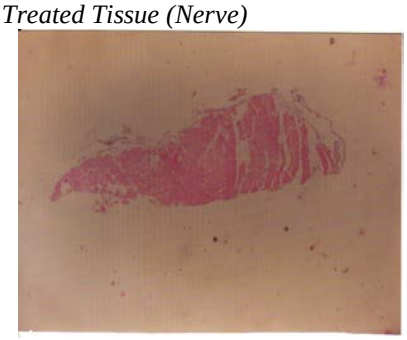


X100

Plate 3. Photomicrographs of a section of the distal end of the sciatic nerve of the toad (0% petrol) (H&E stain).

Treated Tissue (Nerve)



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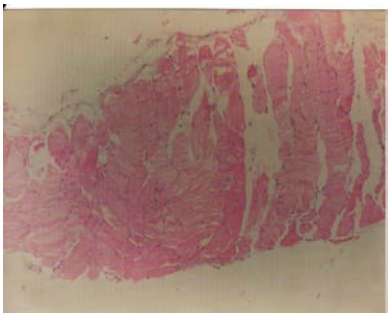

X100

Plate 4. Photomicrographs of a section of the distal end of the sciatic nerve of the toad (2.0% petrol) (H&E stain).

tissue processor has 12 beakers with chemicals for proper tissue processing. In the automatic tissue processor, tissues undergo dehydration using alcohol, clearance using xylene, and finally impregnation. Embedding followed using moulds to give the tissue firmness after solidifying for easy sectioning using the microtome. The sections were finally stained using H & E stain. The stained sections were studied under the microscope for structural changes and photomicrographs of various sections were made.

Statistical Analysis: Data were analyzed using the Analysis of Variance (ANOVA) and presented as mean ± Standard Error of the mean (SEM). The level of significance of the overall effect of gasoline was placed at 95% confidence.

RESULTS

Effect of petrol contamination on threshold voltage, maximal voltage and Latent period: The mean threshold voltages and maximal voltages of stimulation of the sciatic nerve - gastrocnemius muscle preparation are shown in Fig 1. The mean threshold voltages following gasoline contamination were not significantly different from the control (0.15 ± 0.001volts) at various levels of gasoline contamination. The maximal voltage was lower at 0.8% gasoline concentration (0.225±0.014V cf 0.250±0.011V) though this was not statistically significant.

Effect of petrol contamination on Latent period: The latent period is measured as the time lapse between the stimulation and onset of recordable contraction of the nerve-muscle preparation. The mean latent periods obtained for the different levels of gasoline concentration were also not significantly different (figure 2.).

Effect of Petrol on Contraction period: The results for the effects of gasoline concentration on mean contraction time is given in Table 1. Mean contraction period appeared to increase at low concentration of petrol (0.4%) and falls gradually with further increase in concentration, though not significant except at 2% gasoline ($p<0.05$).

Effect of petrol on Relaxation Period: The mean relaxation period for muscle was not significantly changed though slightly increased at 0.4% was reduced at higher concentrations. This result is shown in Table 1.

Effect of Petrol on magnitude of isotonic contractions: The effect of petrol on the mean height of contraction for different levels of concentration is shown in figure 3. Comparisons between the groups show that there was no significant effect between 0.4% petrol and control while there appeared to be a reduction in contractility at the higher concentrations (0.8%, 1.2%, 1.6% and 2.0%; $P<0.05$.). Fig.3.

Effect of Petrol on Velocity of shortening: The velocity of shortening is calculated as the gradient of the contraction curve from the tracings i.e. Difference in y/Difference in x. The result is given in m/sec. The results show an increase at lower concentration and a decrease at higher concentrations. Figure 4.

Effect of Petrol contamination on the absolute refractory Period: The absolute refractory period of the gastrocnemius-sciatic nerve preparation was determined at 22.5º. The mean absolute refractory period increased across the groups. The absolute refractive period was highest at 2.0% concentration; however the increases were not statistically significant. Figure 5.

Results Of Histological Studies: The effect of exposure to varying concentrations of gasoline in amphibian Ringer on the morphology of nerve and muscle is shown in the plates below. The sections were stained using Haematoxylin and Eosin[23]. Plates 1 and 2 are photomicrographs of the cross section of the gastrocnemius muscle for a control tissue under x40, and

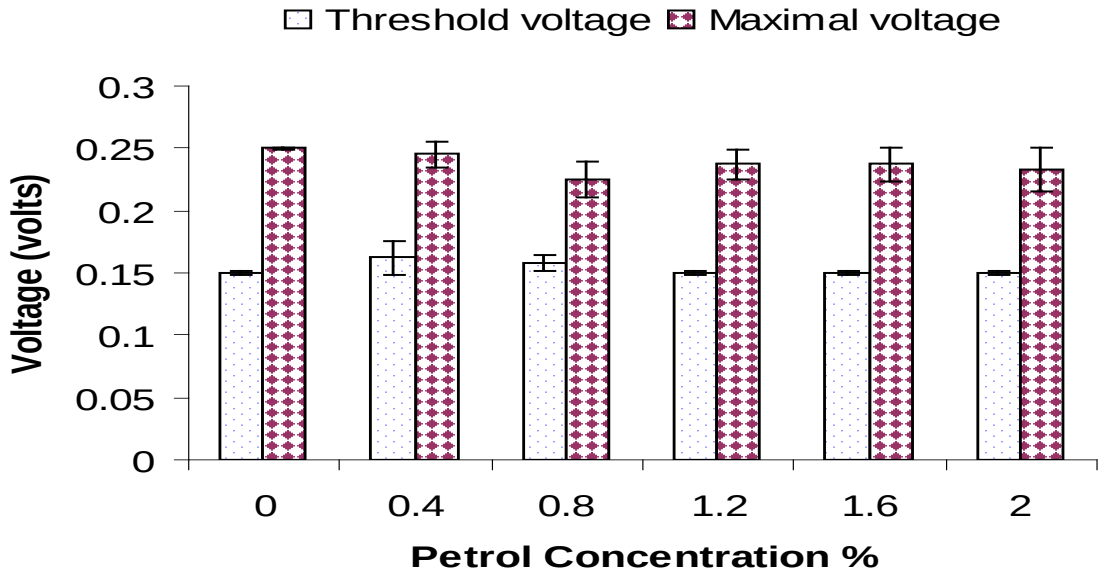

Figure. 1. Effect of varying concentration of petrol on threshold and maximal voltages for the nerve-muscle preparation. Data is presented as mean ± SEM. n = 5.

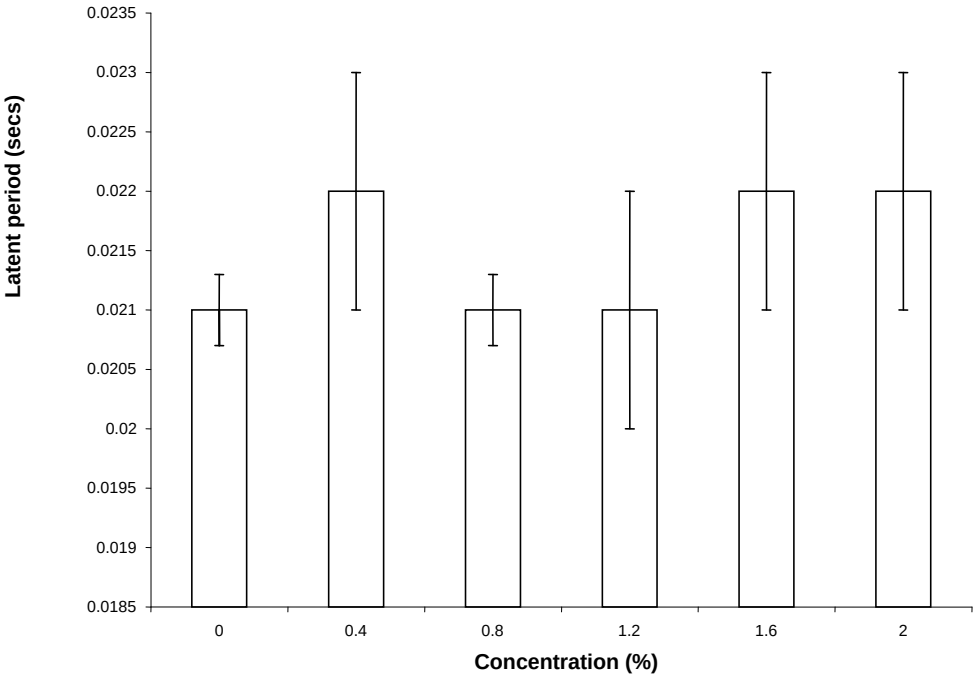

Figure 2: Showing the effect of varying concentrations of petrol on latent period for nerve-muscle preparation

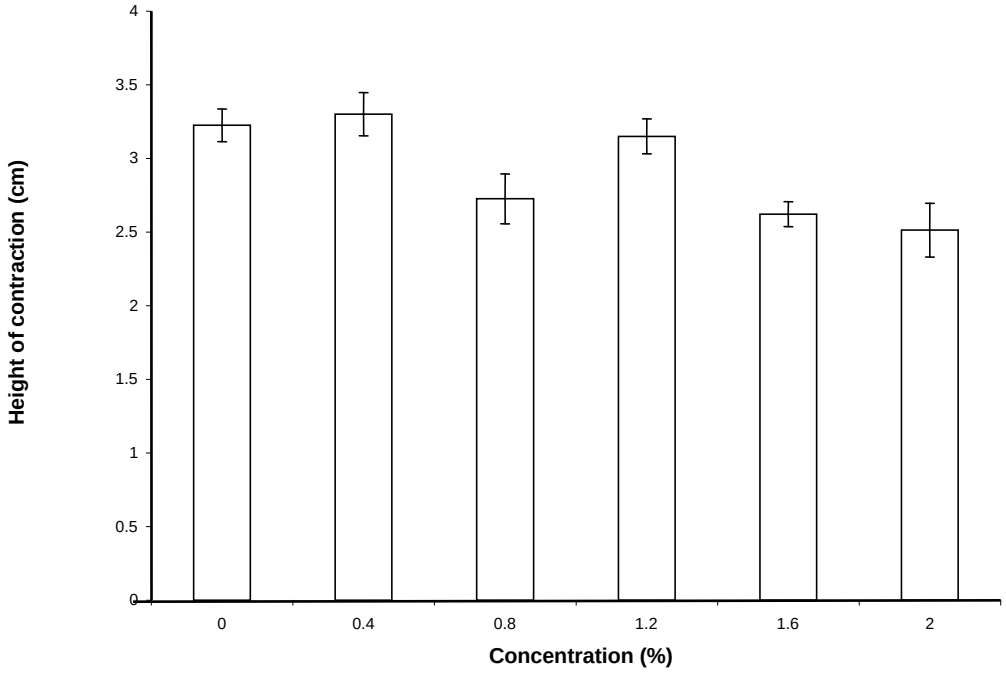

Figure 3: Showing the effect of varying concentration of petrol on height of contraction for the nerve muscle preparation.

x400 magnification. Comparison with the treated tissues at 0.4%, 0.8%, 1.6% and 2.0% show varying degree of oedema. There was tissue damage in the petrol treated tissues. The sections show varying degrees of oedema which may have occurred as a result of tissue injury and inflammation. Sections of the sciatic nerve (control) tissue and petroleum exposed tissue are shown on Plates 3 and 4. The treated groups also show signs of oedema in the nerve sections. The higher concentrations show signs of disintegration or disorganization of tissue matrix. Boundaries are diffuse and less distinct as in the control group.

DISCUSSION

had stated that activity produced little change in the

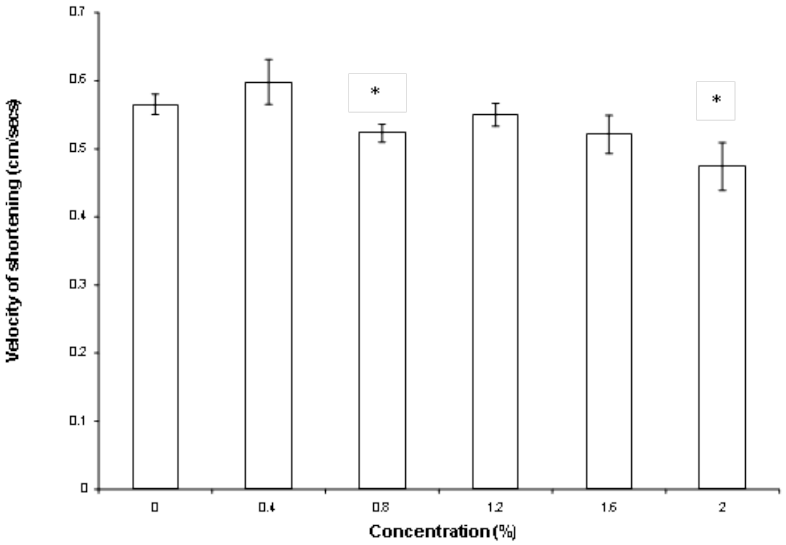

Figure 4: Showing the effect of varying concentration of petrol on velocity of shortening for the nerve muscle preparation. n= 5, * de

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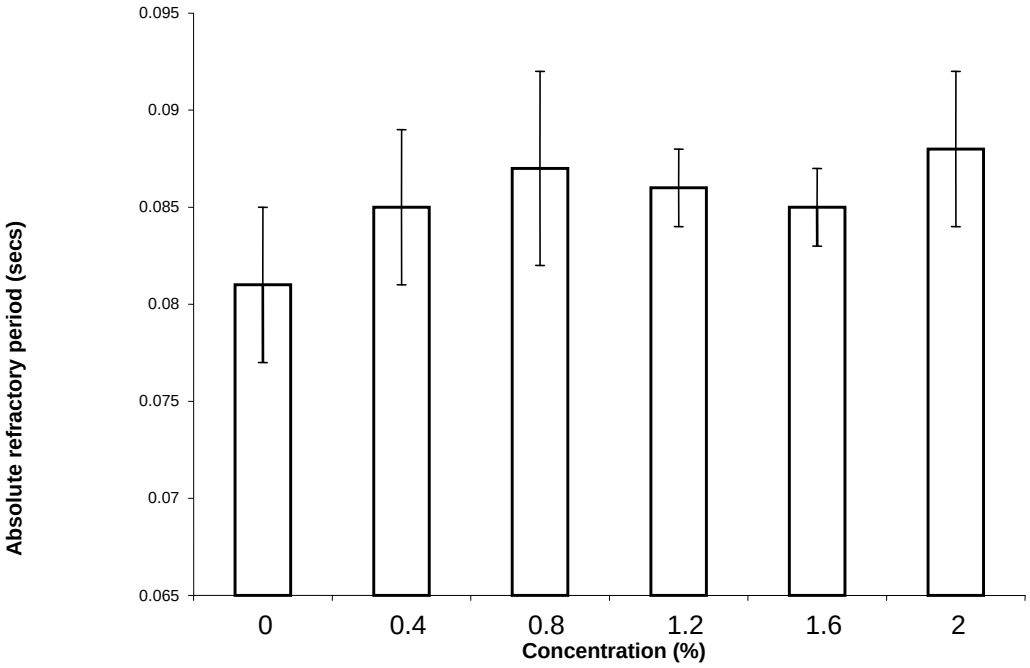

Figure . 5. Showing the effect of varying concentration of petrol in amphibian Ringer on absolute refractory period for the nerve muscle preparation. n = 5. Values are means ± SEM.
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This study assessed the effects of petroleum contamination on neuromuscular function and morphology isolated nerve-muscle preparation using on sciatic nerve and gastrocnemius muscle of the toad. The threshold and maximal voltage remained fairly constant for the different concentrations, however an increase in threshold voltage was observed among two of the treated groups, although these were not consistent. Raymond [24] threshold curve during the refractory period. The *in vitro* studies show that while threshold voltage does not change with increased concentration for most tissues, there is evidence that there can be cytotoxic effects of excessive Ca^{2+} entry in some tissues[25]. The maximal voltage is also not significantly affected by an increase in concentration, although there may be changes in muscle tone.

There was apparently little or no change in the latent period with increasing concentrations of petrol. This may

imply that conduction velocity along the nerve may not have been significantly altered by petrol. This may be due to the fact that most of the sciatic nerve may not be immersed in the contaminated Ringer as it was hung over the stimulating electrodes. An increase in latent period often coincided with a decrease in muscle contractility and a decrease in latent period translates into increase in contractility.

Contraction period, velocity of shortening and height of contraction showed little or no change. As concentration of gasoline increased motor activity increased slightly at first however further increases did not lead to corresponding increase in motor activity? Reiter[25] had reported that motor activity following exposure to Neurotoxic agents result in qualitative and quantitative changes that can be observed using appropriate equipment. The complexity of motor activity is emphasized by the finding that low level exposure to volatile organic solvents increases activity whereas high level exposure decreases it [16]. This dual effect may manifest in this study.

The results follow a trend that shows a significant effect on the contraction parameters. Contraction period, velocity of shortening and height of contraction had considerable level of significance. As concentration increased from 0.0% to 0.4% gasoline, motor activity increased slightly as shown in figures 4 and 6 Further increase however didn't lead to a corresponding increase in motor activity. Reiter[25] had reported that motor activity following exposure to neurotoxic agents result in qualitative and quantitative changes that can be observed depending on the apparatus used. The complexity of motor activity is emphasized by finding that low level exposure to volatile organic solvents increases activity whereas high level exposure decreases it[16]. Earlier studies that have been carried out involving nerve muscle relationships share similar trends with these results. While the results of these previous studies on this subject have varying methods, the results portray similar implications. These implications are a concern to those who have their tissues come in contact with gasoline. OECD publication in 2004 reported that adverse changes in structure or function of the nervous system may result from single or repeated exposures to a chemical[26](OECD 2004). Bessou[27]reported that repetitive stimulation of some single motor axons elicited both the contraction of extrafusal fibres and an increase in the rate of discharge at primary endings. This was further proved in this study by making repetitive stimulus recordings at different concentration using the lowest speed. The results show a sharp variation in contractility among the treated group compared with the control group.

The absolute refractory period is accepted as a standard for neurotoxicity testing in humans [18]. In this investigation of the sciatic nerve and gastrocnemius muscle of the toad the absolute refractory period did not show significant alteration. Chu et al [28] investigated the inhalation toxicity of an ethanol-gasoline mixture in rats and concluded that treatment with ethanol and gasoline, at the levels studied, produced mild, reversible biochemical hematological and histological changes. Morphology in the adrenal gland was characterized by vacuolation of the cortical area. Although histological changes were generally mild in male and female rats and these changes were reversed after 4 weeks. In the present study, sections of the sciatic nerve showed structural disintegration. This gross observation in the treated group is likely due to the lipid layer being dissolved due to its exposure to gasoline. Altered cholesterol levels have been implicated in many neurodegenerative disorders[4], changes in cholesterol levels have also been linked to Alzheimer's disease[6]. Fox et al [13] reported that toxic agents exhibit a preference for distal ends of long peripheral nerves, causing a dying back neuropathy or distal axonopathy. The sections investigated were taken from the distal end of the sciatic nerve. The evidence of morphological distortion was seen in the treated group. This buttresses the findings of Lowndes and Baker[14] that the motor nerve terminals, primary afferent terminals have been compromised in conduction parameters when exposed to gasoline.

Histopathology examination confirmed that there was an interference with the physiological function of the plasma membrane of nerve and muscle which regulate the movement of fluid in and out of the cell. Consequently there was evidence of oedema. The results of these findings confirm that with the techniques used; not only can the presence of neurotoxicity be detected but the site of neurotoxic action also confirmed. An ultra structural study may be required and is recommended as it will help in characterizing the subcellular effects of petrol. It is concluded that gasoline exposure interfered with normal functions of nerves and muscles and also caused structural aberrations in the tissues thus confirming its myo-neural toxicity.

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