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Toxicity of triethyllead chloride (TriEL) on cytoplasmic shuttle streaming, structure, growth and migration of the plasmodial slime mold *Physarum polycephalum*.

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Abstract

Lead is one of the poisonous heavy metals that affects the biotic system. In this research, the toxic effects of different concentrations of triethyllead chloride(TriEL) was studied on the acellular slime mold *Physarum polycephalum* cytoplasmic shuttle streaming, growth, structure and migration of *Physarum* plasmodium. The plasmodium was treated with different concentrations of TriEL (10, 20, 30, 40, 50 and 60 µM). The results showed that concentrations of TriEL<10 µM had no obvious effect on the studied parameters. Initial or slight signs of toxicity appeared at Concentrations between 20 - 50 µM. However, 50 µM TriELwas sublethal. They caused mostly irreversible condensation of the plasmodial strands, blebbing of the plasma membrane, vacuolization of the cytoplasm and elongation of the cytoplasmic shuttle streaming period. 60 µM TriEL was lethal and caused irreversible high blebbing of the plasma membrane, direct and rapid stop of cytoplasmic streaming followed by contraction of the whole plasmodium, strong blebbing of the plasma memmbrane, depigmentation, vacuolization and complete fixation of the cytoplasm (neither growth nor migration occurred on nutrient agar plates).

الملخص

يعتبر الرصاص من العناصر الثقيلة السامة التي تؤثر على النظام الحيوي. تم في هذا البحث دراسة التأثير السام لتراكيز مختلفة من ثلاثي إثيل الرصاص الكلوريدي (TriEl) على الفطر الهلامي اللاخلوي فيزاروم بوليسيفالوم. وشملت الدراسة التأثير على الانسياب السيتوبلازمي الرتيب، النمو، التركيب والزحف للفطر المدروس. تم معاملة طور البلازموديوم بتراكيز من TriEl (60,50,40,30,20,10 ميكرومول). أظهرت النتائج أن تركيز 10 ميكرومول أو أقل ليس له تأثير واضح على الظواهر المدروسة. أما التراكيز بين 20-50 ميكرومول فكانت غير قاتلة حيث أنها سببت إنقباضاً غير منعكس للعروق البروتوبلازمية، تكون الفقاعات في الغشاء البلازمي، زيادة الحويصلات والفراغات في السيتوبلازم والزيادة في مدة دورة الانسياب السيتوبلازمي الرتيب. أما التراكيز 60 ميكرومول فكان التركيز القاتل للفطر حيث تسبب بتلف وتفقع غير منعكس للغشاء الخلوي، توقف مباشر للانسياب السيتوبلازمي، تبعه إنقباض كامل للبلازموديوم وانتشار للصبغة إلى الخارج وزيادة عدد الفقاعات في السيتوبلازم. ولم يظهر البلازموديوم أي شكل من أشكال النمو أو الحركة على أطباق بتري الغذائية.

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Keywords: *Physarum polycephalum* ,triethyllead chloride, cytoplasmic shuttle streaming, lethal dose, plasmodium .

1. Introduction

The last few years gave direct attention to studies that concern with the effect of toxic heavy metals found in the ecosystem. The toxic metals come to ecosystem from various pollution resources such as smoking, cars smoke, sewage, fires and variable resources that use those toxic metals in manufacturing processes(Gloag, 1981; WHO, 1977). TriEL at substantially lower concentrations is able to bring about drastic changes in the organization of intermediate filaments system of mammalian cells (Zimmerman *et al*., 1986). TriEL interacts and disrupts microtubules isolated from mammalian cells(Roderer, 1984). Moreover, TriEL inhibits cell proliferation of normal human lymphocytes (Stiakaki *et al*. 1997), and was found to have erythrocyte haemolytic activity(Kleszcynska *et al*. 1997). The random motility of cells was not markedly inhibited by TriEL, whereas chemotaxis directed cellular movements was strongly inhibited (Zimmerman *et al*., 1986).

Few studies were done concerning the effect of pollution with heavy metals on soil microorganisms.Terayama *et. al*.(1978) studied the toxicity of heavy metals and insecticides on slime mold *Physarum polycephlum*. Wang *et. al*.(2006), and Zeng *et. al*.(2006) discussed the influence soil heavy metals pollution on soil microbial biomass and enzyme activities. Also, Skerving (1993), Bressler and Goldstein (1991) showed that lead is able to inhibit or mimic the actions of calcium, and to interact with proteins (including those with sulfhydryl, amine, phosphate, and carboxyl groups). Shraideh (1999) showed that TriEL induced dramatic changes in the ileum contractile activity in mice.

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There is still a need for detailed studies concerning the physiological and ultrastructural effects of lead on significant microorganisms inhabiting our environment.

The acellular slime mold, *Physarum polycephalum*, represents a suitable model for studying the effect of environmental pollutants,i.e,lead compounds on motility, behavior and ultrastructure of living organisms. Acellular slime molds or myxomycetes represents a strange group of microorganisms. They show plant and animal like characteristics.

Physarum polycephalum is characterized by having a phagotrophic somatic phase (phaneroplasmodium), which is a yellowish, creeping, multinucleated mass of protoplasm enveloped by a slimy sheath and differentiated into two main regions known as massive front and a posterior network of connected veins (fig.1) (Wohlfarth-Bottermann, 1979). These veins which are seen under the light microscope consist of ectoplasm (gel) and a flowing endoplasm (sol), which exhibits a rhythmic shuttle streaming. In addition, the motive force generation in *Physarum* is based on cytoplasmic actin-myosin interaction similar to that of smooth muscles of higher organisms (Kessler, 1982).

The present research concentrated on the following objectives:

A-Studying the effect of different concentrations of TriEL on *Physarum polycephalum*.

B-Investigation of structural and morphological effects of TriEL on phaneroplasmodia of *Physarum polycephalum*.

In studying the effect of TriEL on *Physarum polycephalum* plasmodia, four criteria were taken into consideration:

1. Microscopic responses of whole plasmodium (*In vivo*).
2. Effect on plasmodial migration.
3. Effect on plasmodial structure and shuttle streaming periodicity.
4. Effect on plasmodial growth and viability.

2. Materials and Methods

2.1. Study object

Phaneroplasmodium of *Physarum polycephalum*.(Jordanian isolate similar to ATCC 44912) was used in this study(Shraideh, 1988).

2.2. Effect of test solutions on cytoplasmic shuttle streaming

The shuttle streaming (protoplasmic streaming) is an energy-requiring process. The rate of protoplasmic streaming can be extremely fast (over 1 mm per second) and the duration of an entire cycle of the shuttle streaming is 1.5-3.0 min .Its rhythmicity is a result of its reverse direction every few minutes.

Protoplasmic streaming of phaneroplasmodium of *Physarum polycephalum* (starved on 1.5 % non-nutrient agar media for 24 h) was carried out after submersion of plasmodia in a physiological salt solution (PSS) as control for about 10 min. PSS conisted of: 6.0 mM NaCl, 3.0 mM KCl, 1.0 mM $CaCl_2$, 0.1 mM $NaHCO_3$, 0.5 mM $MgCl_2$. Submerged plasmodium in PSS was observed under a phase contrast microscope (at 100X and 400X magnifications). After 5-10 min. adaptation (when streaming period was nearly stable), shuttle streaming periods were recorded and followed for about 2 hs (Shraideh, 2006).

The PSS solution was removed and replaced by different concentrations (10, 20, 30, 40, 50,& 60 µM) of TriEL (TriEL was obtained from Ventron, Karlsruhe, Germany). After 5-10 min. submersion, shuttle streaming periods were recorded and followed. For every tested solution, 30 periods were measured, the mean and standard deviation were calculated (Table 2) . Recorded values were compared with that of a control plasmodium (streaming and morphology under PSS). Also morphological changes were observed at least for 2 h. Depigmentation, the release of pigments and decoloration of plasmodia were observed by the yellowish coloration of submersion solution.

2.3. Effect of test solutions on growth of Physarum plasmodium:

Corn- agar plates were used for growth test. Corn-agar medium consisted of: 6.0 gram rolled oats+1.0 gram D-glucose+7.5 gram agar- agar +500 ml distilled water. Contents were mixed, sterilized in autoclave at 121°C for 20 min and poured in sterile plates. Petri dishes were prepared including growth media (control) and successive concentrations of TriEL (10, 20, 30, 40, 50 and 60 µM), which were added individually to the growth medium. Small pieces of *Physarum* plasmodia were allowed to grow on medium surface and growth was observed and sketched at intervals of 2h (Shraideh, 2006).

2.4. Viability test:

Viability of phaneroplasmodium of *Physarum polycephalum* (starved on 1.5% non–nutrient agar medium for 24 hours) was studied after treating with two different concentrations (50 and 60µM) of TriEL for 4 h. Treated plasmodia were transferred to 1.5 % agar-agar plates and kept overnight. Their ability to make phaneroplasmodia was compared to that of control (PSS treated plasmodia)(Korohoda *et. al*., 1983).

2.5. Effect of test solutions on migration of Physarum plasmodium

1.5% non-nutrient agar medium was used for migration test. Petri dishes were prepared as follows:

- A-1.5% non-nutrient agar media (control).
- B-Successive concentrations of TriEL were added to 1.5% non-nutrient agar medium (migration test).

Small pieces of *Physarum* plasmodia were allowed to migrate on the surface of the agar plates. Migrating plasmodia were sketched at intervals of 2h.

2.6. Time lapse phase contrast photomicrography:

The effect of two concentrations (50, 60µM) of TriEL on phaneroplasmodia of *Physarum polycephalum* (starved on 1.5 % non-nutrient agar media for 24 h) was followed using Zeiss stereophotomicroscope. Photography was started under PSS at 0- time (few min. after submersion of the plasmodium with PSS), then with the test solutions(50, 60µM of TriEL) at 10 min. intervals.

All experiments of this research were done at a temperature of 24-26°C.

3. Results

3.1. Effect of TriEL on Physarum polycephalum phaneroplasmodia

The effects of different concentrations of TriEL on whole phaneroplasmodia migrating on agar surface are summarized in table (1), which shows the sequence of events following the treatment of the plasmodia with different cocentrations of TriEL.

3.2. Effect of TriEL on plasmodial migration:

The effect of different concentrations of TriEL on migration ability of *Physarum* plasmodia on non-nutrient agar was investigated. Migration of the plasmodia was followed overnight and sketched at intervals of 2 h. The results are shown in Figure 2. Plasmodia transferred into TriEL media were compared to those on control media. The results showed that:

1. In B1(Plasmodium transferred into 10 µM TriEL media) there were no obvious changes in migration.
2. In B2, B3, B4(Plasmodium transferred into [20-40 µM TriEL media) showed gradient decrease in migration ability.
3. In B5(Plasmodium transferred into sublethal dose [50 µM TriEL] media) showed a very short distance migration ability.
4. In B6(Plasmodium transferred into lethal dose [60 µM TriEL] media) showed no migration ability at all.

3.3. Effect of TriEL on the viability of Physarum plasmodia:

Treatment of plasmodia with (50 and 60 µM) of TriEL affected their viability. The results are shown in Figure 3. Plasmodia treated with 50 µM of TriEL showed very weak spreading ability compared with control. While treatment with 60 µM TriEL resulted in complete inhibition of phaneroplasmodia

3.4. Effect of TriEL on plasmodial structure& shuttle streaming periodicity:

The protoplasmic strand of *Physarum* in a cross section is composed of 2 distnct regions; ectoplasm and endoplasm (figure 1). The more viscous or gel ectoplasm represents about 80% of volume of the protoplasm. It is rich in labyrinth of invaginations from which vesicles are pinched off to the inside. Contractile vacuoles and other kinds of vacuoles are present. The endoplasm, which is the central part is less viscous and includes streaming nuclei, mitochondria, ribosomes, pigment granules, vacuoles, vesicles and other organelles. The protoplasm in *Physarum* plsmodial strands(veins)exhibits reversible or regular shuttle streaming with period duration of approximately 1.3-3.0 min.(Shraideh, 1988).

The endoplasm(inside stationary ectoplasm) streams in one direction for a short while, stops and then turns back for a while and so on.

Table 2 summarizes the effect of TriEL on shuttle streaming periodicity.

The effects of a sublethal concentration (50 µM) and the lethal concentration (60 µM) of TriEL were investigated on the structure and behaviour of treated phaneroplasmodia (fig.4). After half an hour of addition of the lethal concentration of TriEL only vacuolization of endoplasm occurred. After 1 hour darkening and more vacuolization were observed. After 1.25 hour addition of the lethal concentration, high vacuolization, blebbing of plasma membrane and stop of shuttle streaming occurred.

By comparing results with control it was found that the lethal concentration of TriEL caused complete fixation and disruption of the plasmodium after 1.5 hours of treatment.

3.5. Effect of TriEL on growth of Physarum polycephalum:

Mixing the corn agar media with the different concentrations (10-60 µM)TriEL affected the growth of *Physarum* plasmodia. The effect of TriEL was investigated and reported as sketches.

Figure 5 shows the results obtained. Plasmodia transferred into TriEL-media were compared to those transferred to control media. The results showed that in B1-B5 (Plasmodium transferred to 10,20,30,40 or 50 µM TriEL media) there was a gradient decrease in migration ability. 50 µM TriEL medium resulted in a very short distance migration, that was highly inhibited after 3 h. While in B6 (plasmodium transferred into the lethal concentration (60 µM)TriEL), the results showed no migration ability at all, with extreme depigmentation.

Table 1.Effect of gradient concentrations of TriEL on *Physarum* whole plasmodia.

Concentrations [µM TriEL / ml]	Effect
PSS(control)	Plasmodium showed normal migration, small vacuoles in streaming endoplasm. Stationary ectoplasm.
10.0	Plasmodium has good migration ability but darker in color. The plasmodium slowed down streaming velocity, with a low degree of vacuolization.
20.0-30.0	Slow migration ability of plasmodium was noticed.. Vacuolization, depigmentation and contraction of the frontal region was noticed after 2h of treatment.
40.0-50.0	Condensation of the whole plasmodium. Decolorization and release of pigments. Very little migration ability was observed.
60.0	Direct stop of cytoplasmic streaming. No migration ability.Condensation, depigmentation of plasmodium was observed after 2 h of treatment.

Table 2 .Effect of gradient concentrations of TriEL on plasmodial shuttle streaming periodicity and plasmodial structure.

Treatment	Streaming Period				Observations
	Mean ± SEM	Percentage	n	N	
PSS	2.07±0.03 min	100.0%	30	3	Regular streaming, no large vacuoles.
10µM	2.23±0.04 min	107.7%	30	3	Regular streaming. Little effect on period duration.
20µM	2.91±0.04 min	140.6%	30	3	Elongation in streaming period and little vacuolization.
30µM	2.95±0.03 min	142.5%	30	3	Elongation in streaming period, obvious vacuolization.
40µM	3.02±0.08 min	145.9%	30	3	Blebbing and disturbance occurred in large veins. Difficulty to monitor the shuttle-streaming period.
50µM Sublethal concentration	----------	----------	---	4	Very long shuttle streaming periods (up to 5 min) or sometimes very short periods (up to59-sec.). Blebbing was obvious.
60µM Lethal concentration	----------	----------	---	4	Immediate stop of cytoplasmic streaming followed by decolorization. Viability test showed no migration ability.(irreversible effect)

*n: number of periods analyzed.
*N: number of plasmodia used.
*Percentage: Mean of streaming periods of treated plasmodia/ Mean of streaming periods of the control (100%)
*SEM: Standard error of mean

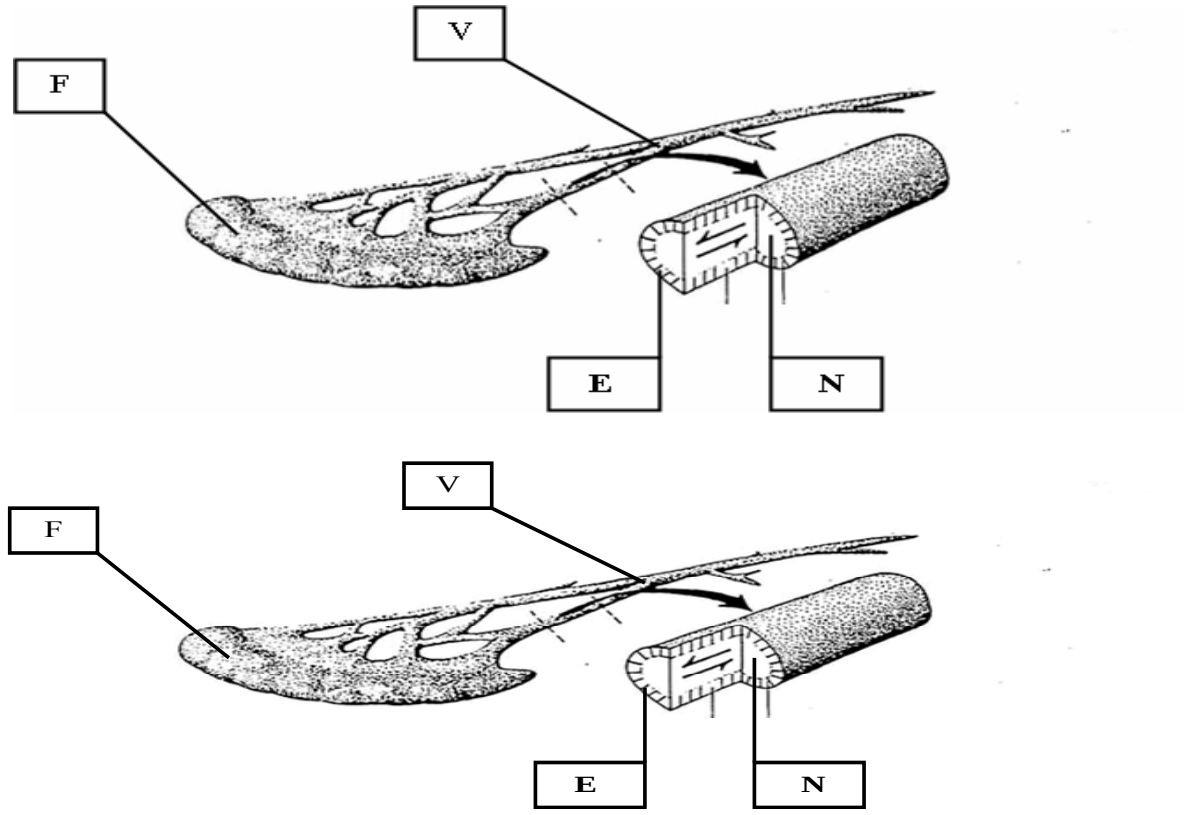

Figure 1: Plasmodium of *Physarum polycephalum*

F: Front , V: Veins, E: Ectoplasm and N: Endoplasm. (Adopted from Wohlfarth-Bottermann, 1979)

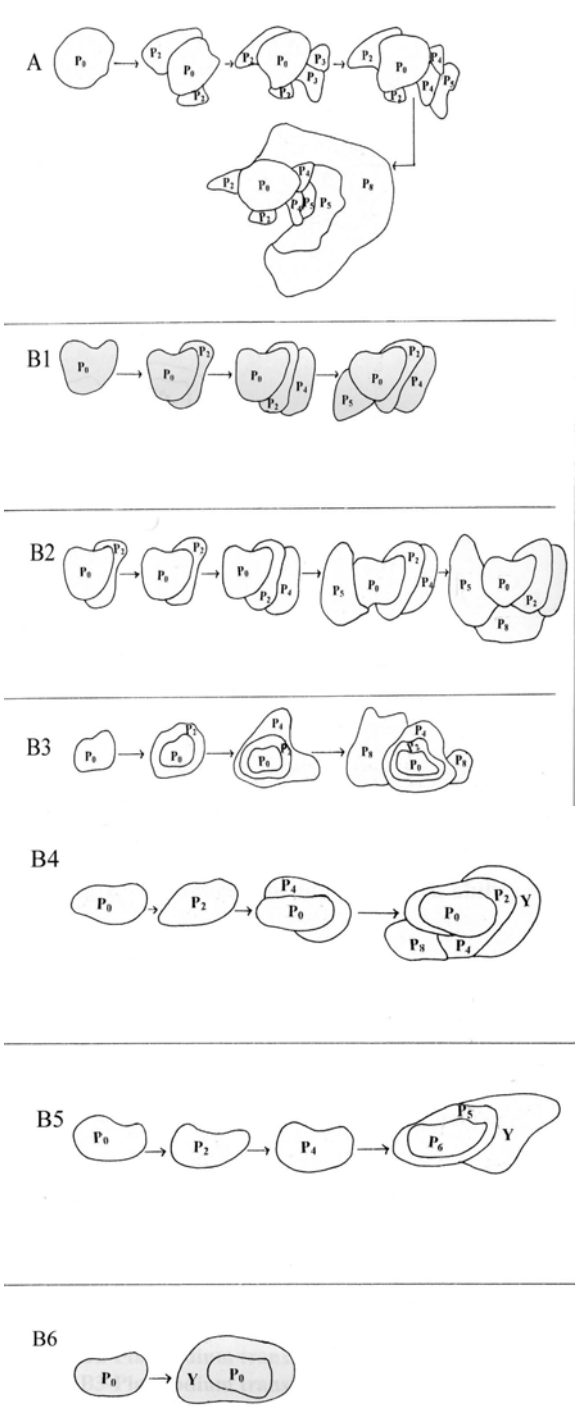

Figure 2. Sketches showing migration ability of plasmodia treated with TriEL.
P_0:Codes for plasmodium at zero time.
P_2:Codes for plasmodium after 2 h.
P_3:Codes for plasmodium after 4 h.
P_5:Codes for plasmodium after 6 h.
P_8:Codes for plasmodium after 8 h.
Y:Yellow color caused by depigmentation.
A-Plasmodium transferred to 1.5% agar medium (control).
B-Plasmodium transferred into 1.5%agar medium containing gradient concentrations of TriEL:
B1-Plasmodium transferred into 10 µM TriEL medium.
B2-Plasmodium transferred into 20 µM TriEL medium.
B3-Plasmodium transferred into 30 µM TriEL medium.
B4-Plasmodium transferred into 40 µM TriEL medium.
B5-Plasmodium transferred into 50 µM TriEL medium (Sublethal concentration).
B6-Plasmodium transferred into 60 µM TriEL medium (Lethal concentration).

4. Discussion

This study is one of few studies concerning the effect of environmental contamination with heavy metals on slime molds inhabiting our environment. In this study we investigated the effect of different concentrations of TriEL on different parameters of life of the acellular slime mold *Physarum polycephalum* isolated from a north forest in Jordan (Shraideh, 1988).

The results showed that concentrations of TriEL (10, 20, 30, 40 and 50 µM) represent the sublethal concentrations while the concentration (60 µM) represents the lethal concentration, which affected the activity, structure and life of the acellular slime mold *Physarum polycephalum.*

TriEL concentration of 10 µM of TriEL caused a little effect on structure or streaming of the cytoplasm. TriEL concentrations of 20 and 30 µM caused vacuolization of the cytoplasm, increasing the streaming period about 40%, and very low ability of migration.While 40 and 50 µM induced high vacuolization and condensation of the cytoplasm, blebbing of plasmalemma and very long streaming periods.On the other hand, the lethal concentration (60 µM) caused an immediate stop of cytoplasmic streaming, followed by depigmentation of plasmodium. Similar effects were observed from treatment of *Physarum* plasmodia with CdCl2 and PbCl2 (Shraideh 2006, 2007 respectively).

From these structural observations we can conclude that TriEL affected the integrity of the biological membranes. The weakening of the membrane led to the observed effects i.e. vacuolization of the cytoplasm, blebbing of plasmalemma and its disruption. Terayama *et. al*.(1978) suggested that the toxicity of heavy metals in the slime mold *Physarum polycephalum* is accompanied by some changes in the cell membrane. could be also explained by the ability of Lead can bind membrane proteins (Skerving, 1993) and is able to substitute Ca^{++}(Bressler and Goldstein, 1991) which causes disturbance in permeability of plasma membrane and normal function of membrane proteins. This may explain the observed weakening of membrane, blebbing and loss of pigments in the TriEL-treated plasmodia.

The internal organization of protoplasmic strands (veins) and the presence of actomyosin fibrils in the ectoplasm of the strand are important for shuttle streaming, migration and growth of the plasmodium (Wohlfarth-Bottermann, 1979). Disruption of internal

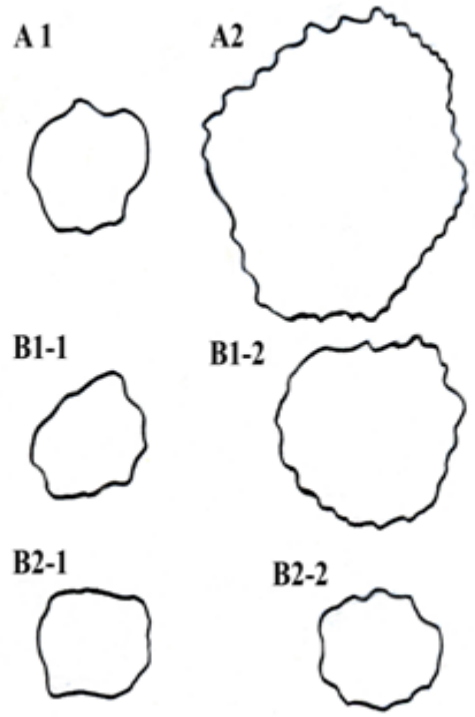

Figure 3. Sketches showing effect of TriEl on viability of *Physarum* plasmodia

A-Control plasmodium at:
A_1: Zero time. A_2: After 4 h.
B-Plasmodium treated with TriEL concentrations of:
B_1: Sublethal concentration (50 µM):
B_{1-1}: Sublethal concentration at zero time.
B_{1-2}: After 4 h treatment with the sublethal concentration.
B_2: Lethal concentration (60 µM):
B_{2-1}: Lethal concentration at zero time.
B_{2-2}: After 4 h treatment with the lethal concentration.
Magnification = 6 X.

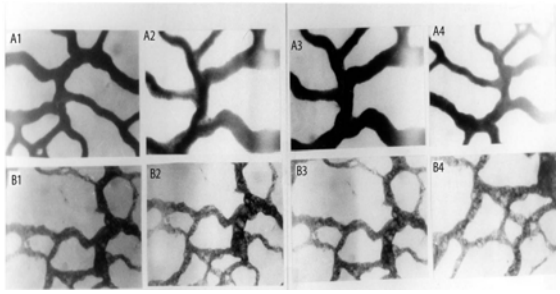

Figure 4. Time laps photomicrographs showing effect of TriEL on the shuttle streaming and structure of *Physarum* phaneroplasmodium.

A-Control plasmodium (PSS solution). Note normal structure of protoplasmic strand.
A_1: After 30 min.
A_2: After 60 min.
A_3: After 75 min.
A_4: After 90 min.
B-Plasmodium treated with TriEL lethal concentration (60 µM)
B_1: After 30 min. There was a slight vacuolization of cytoplasm.
B_2: After 60 min. Condensation and more vacuolization of cytoplasm was observed.
B_3: After 75 min. High condensation, extensive vacuolization of cytoplasm and
blebbing of plasmalemma was observed.
B_4: After 90 min. Condensation and extensive vacuolization of cytoplasm, blebbing of plasmalemma were observed. Also disruption of structural organization of plasmodium was obvious.
Magnification = 5X

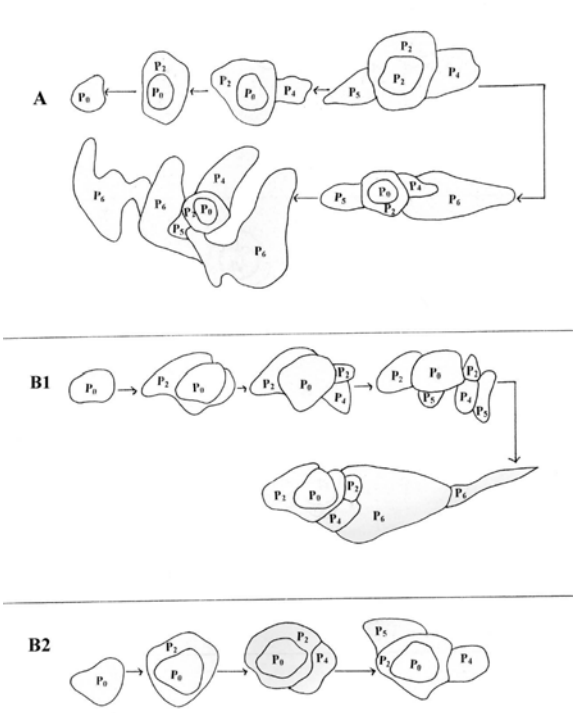

Fig 5. Sketches showing growth ability of plasmodia treated with TriEL.

P_0: Codes for plasmodium at zero time.
P_2: Codes for plasmodium after 2 h.
P_3: Codes for plasmodium after 3 h.
P_4: Codes for plasmodium after 4 h.
P_6: Codes for plasmodium after 6 h.
P_8: Codes for plasmodium after 8 h.
Y: Yellow color caused by depigmentation.
A-Plasmodium transferred into corn agar medium (Control).
B-Plasmodium transferred into growth media containing gradient concentrations of TriEL:
B1-Plasmodium transferred into 10 µM TriEL growth medium.
B2-Plasmodium transferred into 20 µM TriEL growth medium.
B3-Plasmodium transferred into 30 µM TriEL growth medium.
B4-Plasmodium transferred into 40 µM TriEL growth medium.
B5-Plasmodium transferred into 50 µM TriEL growth medium (Sublethal concentration).
B6-Plasmodium transferred into 60 µM TriEL growth medium (Lethal concentration).

organization, vacuolization and disturbance of actomyosin fibrils could explain the drastic effects of TriEL treatment These effects range from slow of shuttle streaming, vacuolization and at end by complete irreversible stop of cytoplasmic streaming, loss of pigments and blebbing of plasma membrane.

TriEL has been found to disassemble microtubules in cultured mammalian cells(Zimmermann, et al, 1988) and is able to disrupt microtubules and inhibit motility of *Dictyostelium discoideum*(Sroka et al, 2002). *Physarum* contains a microtubule assembly that supports actin-myosin organization, which is responsible for motility, streaming, and support of plasma membrane(Diggens & Williams, 1987). Treatment of *Physarum* plasmodia with

different concentrations of TriEL may disrupt and disassemble microtubules and cause disorganization of actin-myosin organization. This may explain the results of plasmodial treatment with TriEL, including membrane blebbing, stop of motility and irregular or complete stop of cytoplasmic streaming.

These results are in agreement with those of other studies which showed that lead is able to replace and fuction as Ca^{++} (Bressler and Goldstein, 1991), to inhibit ion channels and to increase membrane permeability (Skerving, 1993).

The inhibitory effect of TriEL on plasmodial growth and migration can also be explained by the findings of Wang, *et. al*. (2006) who showed that heavy metals pollution affected negatively microbial biomass, activity and community composition in soil. Also Malecka, *et. al.* (2001) discussed the ability of lead to bind nucleic acids causing condensation of chromatin, stabilization of the DNA double helix and thus inhibiting the process of replication and transcription. They also showed that lead can exert a negative effect on mitochondria by decreasing the number of mitochondrial cristae, which in turn lowers the capacity of ATP production. This may explain the slow migration and weak growth of lead treated *Physarum* plasmodia. TriEl has been found to affect dramatically the ileum contractile activity in TriEL treated mice (Shraideh, 1999).

Finally we can say that pollution of the environment with heavy metals like lead will have a bad effect on the environmental structure and equilibrium.

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