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# Effects of $Cd^{2+}$, $Zn^{2+}$, $Li^{+}$ and $Al^{3+}$ on the Fertilization-Induced Membrane Potential Changes in the Egg of the Frog Rana *cameranoi*

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**Abstract:** The apparent change in the egg membrane potential at fertilization gives rise to fertilization potential (FP). FP is the initial, transient electrical block to polyspermy as shown in most species especially those exhibiting external fertilization, and it protects the egg from a second sperm entry until the permanent, mechanical block is set up. Polyspermy risk is increased when this effective electrical block cannot reach a certain peak value or when it is of short duration. On the other hand, metal accumulation in the external environment is potentially toxic to living beings, and we aimed in the present study to evaluate the toxic effects of $Cd^{2+}$, $Zn^{2+}$, $Li^{+}$ and $Al^{3+}$ on the potential changes triggered by fertilization in the egg of the frog, Rana *cameranoi*. Egg membrane potentials were recorded using the conventional microelectrode technique. Resting membrane potential before fertilization and FP parameters were determined. Ten percent Ringer solution (pH=7.80) was used as a control, and experimental groups were established by adding $CdCl_2$ (68 µM), $ZnCl_2$ (300 µM), LiCl (11.1 mM) or $AlCl_3$ (1.5 mM) into this solution and adjusting their pHs to 7.80.

Evaluation of our results revealed that duration of potential change induced by fertilization was significantly reduced by $Li^{+}$ and $Al^{3+}$ whereas $Cd^{2+}$ prolonged the duration of FP ($p<0.05$). Peak fertilization potential ($FP_p$) was significantly more positive with $Zn^{2+}$ ($p<0.05$), and this ion did not significantly influence other parameters.

We conclude that especially $Al^{3+}$ and $Li^{+}$, the potential embryotoxic and teratogenic metals, can apparently increase the risk of polyspermy, but $Cd^{2+}$ and $Zn^{2+}$ concentrations used in this study are not hazardous for the reproduction of frogs. The greater accumulation of $Al^{3+}$ and $Li^{+}$ in parallel with industrial development may have serious deleterious effects on the reproduction of species that show external fertilization, and this fact may consequently be harmful for the ecologic equilibrium in the near future.

**Key Words:** Fertilization potential, frog egg, cadmium, zinc, lithium, aluminium

## Rana *cameranoi* Türü Kurbağa Yumurtasında Fertilizasyon ile İndüklenen Membran Potansiyel Değişimlerine $Cd^{2+}$, $Zn^{2+}$, $Li^{+}$ ve $Al^{3+}$'un Etkileri

**Özet:** Fertilizasyonla yumurta zarında belirgin bir potansiyel değişimi (Fertilizasyon Potansiyeli, FP) meydana gelmektedir. FP, özellikle dış döllenme gösteren canlıların bir çoğunda gösterildiği gibi,

polispermiye karşı ilk sırada oluşturulan geçici elektriksel blokdur ve mekanik engel oluşturulana kadar yumurtayı ikinci bir spermin girişine karşı korur. Bu efektif elektriksel blok belli bir pik değere ulaşamaz veya kısa süreli olursa polispermi görülme riski artar. Diğer yandan, dış ortamda artarak devam eden metal birikimi canlılara toksik etki göstermektedir. Bu çalışmada, $Cd^{2+}$, $Zn^{2+}$, $Li^{+}$ ve $Al^{3+}$ gibi metal toksisitesinin fertilizasyonda kayıtlanan potansiyel değişimlere olan etkilerinin incelenmesi amaçlanmıştır. Çukurova Bölgesinde yaygın olarak bulunan Rana *cameranoi* türü kurbağa yumurtalarının membran potansiyelleri mikroelektrod tekniği kullanılarak incelendi. Fertilizasyondan önceki dinlenim membran potansiyeli ile FP parametreleri değerlendirildi. Kontrol olarak %10 Ringer solüsyonu (pH=7.80) kullanılırken bu solüsyona $CdCl_2$ (68 µM), $ZnCl_2$ (300 µM), LiCl (11.1 mM) ve $AlCl_3$ (1.5 mM) eklenerek ve pH'ları 7.80'e tampone edilerek deney grupları oluşturuldu.

Elde edilen verilerin değerlendirilmesi sonucunda, $Li^{+}$ ve $Al^{3+}$'un fertilizasyon ile indüklenen potansiyel değişim sürelerini anlamlı olarak kısalttığı, $Cd^{2+}$'un ise FP süresinde uzamaya neden olduğu saptandı ($p<0.05$). $Zn^{2+}$ daha pozitif pik FP'ne neden olurken ($p<0.05$) diğer parametrelerde kontrol değerleri ile büyük benzerlik gösterdi.

Embriyotoksik ve teratogenik olduğu bilinen metallerden özellikle $Al^{3+}$ ve $Li^{+}$'un polispermi riskini belirgin olarak arttırdığı, bununla beraber çalışılan $Cd^{2+}$ ve $Zn^{2+}$ yoğunluklarının kurbağa üremesi için tehdit oluşturmadığı söylenebilir. Kurbağa gibi dış döllenme gösteren canlıların üremesi üzerinde ciddi olumsuz etkilerde bulunabilen $Al^{3+}$ ve $Li^{+}$'un giderek artan birikimi devam ederse, yakın gelecekte ekolojik dengede önemli bozulmalara yol açabileceği mümkün gözükmektedir.

**Anahtar Sözcükler:** Fertilizasyon potansiyeli, kurbağa yumurtası, kadmiyum, çinko, lityum, aluminyum

## Introduction

Fertilization activates some metabolic and ionic processes in the egg, which result in embryonic development. When activated by a sperm, the egg membrane potential changes suddenly within seconds, and fertilization potential (FP) develops (1,2). FP was demonstrated to be a transient electrical block functioning against polyspermy especially in species reproducing externally (1,2,3,4,5), and it blocks second sperm entry until the genesis of a mechanical barrier (cortical reaction) (1,2,6). FP is functional for nearly 10 min in the frog egg (3,7) and this duration may be as long as 90 min in the nemertean (4). Shortening of FP duration increases the incidence of a lethal process; polyspermy (6). In addition, apparent potential change in the egg membrane triggered by means other than sperm makes the egg unable to be fertilized by the sperm (1,2,6,7,8,9). For this reason, egg resting membrane potential has to be held at optimal levels, and FP has to reach a peak value that lasts until the mechanical block is established; both of these processes possess vital importance for a successful fertilization (1,2).

The first change in the egg when it is activated (by a sperm or artificially) is the increase in ooplasmic $Ca^{2+}$, and this has been demonstrated in all species that were investigated (2,10,11,12,13,14,15,16). Increased ooplasmic $Ca^{2+}$ leads to genesis of FP in the frog, sea urchin, and fucoid alga (2,3,8,9,17,18).

Greater accumulations of heavy metals like cadmium, zinc, lithium and aluminium seem to be inevitable in parallel with industrial development. These metals exert embryotoxic/teratogenic effects by directly or indirectly affecting cellular physiology (19,20,21,22,23). These metals, which have been determined to show cellular toxicity, may also influence the potential changes induced by fertilization. $Cd^{2+}$ and $Zn^{2+}$ interact with divalent cations such as $Ca^{2+}$ (19,24), lithium inhibits the $IP_3$ cycle and consequently the rise in intracellular calcium (25,26), and aluminium finally blocks the second messenger system by possibly acting through the GTPase (27). These actions mentioned above may directly affect the genesis of fertilization potential. In addition to its influence on FP, $Ca^{2+}$ plays the key role in many events triggered by fertilization (e.g. cortical granule exocytosis); it thus seems possible that factors demolishing $Ca^{2+}$ homeostasis can also seriously affect fertilization.

In the present study, we aimed to investigate the effects of external heavy metal accumulation on the events triggered by fertilization in the frog egg, by using the electrophysiologic technique.

## Materials and Methods

Selected frogs of Rana *cameranoi* species were kept until use at +4ºC , in plastic boxes, which were filled with small amounts of stock solution (28).

### Obtaining eggs and spermatozoa; insemination procedure

For induction of ovulation in the sexually mature females, the pituitary glands which were removed from female frogs were homogenated and injected intraperitoneally, and progesterone (Sigma, P-0130) was injected into the thigh muscle (29). The amounts of injected pituitary glands and progesterone were adjusted according to the season (30). The injected frogs were kept at 18ºC for 36 h, or at 25ºC for 24-36 h, and eggs were then obtained by squeezing from the cloaca. Mature eggs were selected according to the criteria cited by Rugh (31).

Sperm suspensions were obtained by macerating frog testis in 10% Ringer, 2-5 h after a male was injected with 300 IU human chorionic gonadotropin (hCG, Sigma CG-2) intraperitoneally. Sperms were examined for motility and morphology under a microscope (Nikon) at 400X magnification.

The insemination procedure was carried out as described earlier (3). Normal fertilization was scored by a shift in membrane potential towards positive values (within 5 min), rotation (at about 30 min), and normal first cleavage (at about 2.5 h) (5). Experiments were performed at 21-25ºC.

### Control and experimental groups

Control records (n=12) were carried out in standard 10% Ringer solution which contained

(in mmol/l): NaCl, 11.1; KCl, 0.19; $CaCl_2$, 0.11; $MgSO_4$, 0.08; NaOH, 0.4; HEPES, 0.25; pH 7.80 (5). Metals were added to the solutions in their LD50 concentrations reported in the literature, as follows: 68µM $CdCl_2$ (Sigma, C-3141), 300 µM $ZnCl_2$ (Sigma, Z-4875), 1.5 mM $AlCl_3$ (Sigma, A-7178) (20,22,32). Since the LD50 value of lithium is high (80 mM) (23) and it might therefore affect the osmolality of the 10% Ringer solution that was used, lithium was substituted for the same amount of NaCl that the control solution included (11.1 mM LiCl). Records obtained in these solutions served as experimental groups. pH values of the metal containing solutions were all adjusted to that of the control solution (pH=7.80).

### Electrophysiological measurements

Intracellular records were obtained by performing the conventional microelectrode technique. The electrode was inserted into the egg by transiently increasing the negative capacitance of the preamplifier (Nihon Kohden MEZ-7200) to produce an oscillating current. Membrane potentials were monitored on a storage oscilloscope (Nihon Kohden VC-10), and recorded on a chart recorder (Palmer Bioscience). The following parameters from the electrical recordings were investigated: resting membrane potential of the unfertilized mature egg (RMP); fertilization time ($F_t$); peak fertilization potential ($FP_p$); duration of effective fertilization potential ($EFP_d$), which is the time from the beginning of fertilization potential through the membrane potential level of –10 mV; duration of fertilization potential ($FP_d$); and membrane potential of the fertilized egg ($MP_f$) (Figure 1).

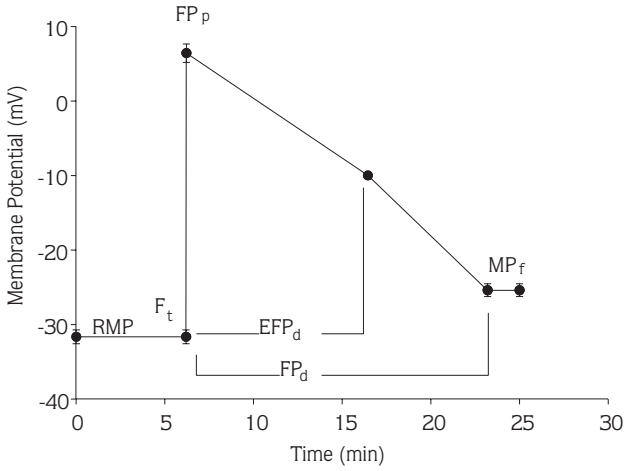


Figure 1. Graphical illustration of evaluated parameters drawn by using mean values (±SEM) determined in the control group (RMP, resting membrane potential; $F_t$, fertilization time; $FP_p$, peak fertilization potential; $EFP_d$, duration of effective fertilization potential; $FP_d$, duration of fertilization potential; $MP_f$, membrane potential of the fertilized egg).

### Statistical analysis

All averages were expressed as the Mean ± SEM. Significance of differences between means was determined using one-way ANOVA tests. The criterion for significance was $p<0.05$.

## Results

A sudden, rapid (within seconds), and depolarizing membrane potential change was recorded in the eggs of Rana *cameranoi* frogs at fertilization. This potential change (Fertilization Potential, FP) lasted for 17.0±1.36 min, and membrane potential of the fertilized egg then recovered to a stabilized level (Fig 1, Fig 2A). Addition of metals to the extracellular solution did not influence RMP significantly, but did affect the properties of bioelectrical potential change induced by fertilization ($p<0.05$, Table, Fig. 2B-E).

**Resting Membrane Potential:** RMP value in the control group was found to be (Mean±SEM) –31.67±0.94 mV. In the experimental groups, RMP did not differ significantly when compared to the control value (Table).

### Fertilization Potential Parameters

**Fertilization Time ($F_t$):** $F_t$ was determined to be 6.20±0.37 min in the control group, and it did not differ significantly in the experimental groups ($p>0.05$, Table).

**Peak Fertilization Potential ($FP_p$):** In the control group, sudden depolarization induced by fertilization reached 6.42±1.23 mV; this value was significantly more positive in the group exposed to $Zn^{2+}$ (17.8±3.16 mV; $p<0.05$) (Table, Fig. 3B). It did not change evidently with

Table. Values of RMP and FP parameters in the control and experimental groups (Mean±SEM).

| Group | RMP (mV) | $F_t$ (min) | $FP_p$ (mV) | $EFP_d$ (min) | $FP_d$ (min) | $MP_f$ (mV) |
|---|---|---|---|---|---|---|
| Control (n=12) | -31.67±0.94 | 6.20±0.37 | 6.42±1.23 | 10.25±1.07 | 17.0±1.36 | -25.42±0.86 |
| $Cd^{2+}$ (n=10) | -36.9±2.62 | 5.28±0.29 | 8.6±1.99 | 7.51±1.30 | 25.49±1.29* | -29.2±1.82 |
| $Zn^{2+}$ (n=10) | -28.33±1.31 | 5.44±0.23 | 17.8±3.16* | 9.16±1.37 | 17.0±1.15 | -23.5 ±1.69 |
| $Al^{3+}$ (n=10) | -28.07±1.35 | 5.15±0.21 | 10.4±1.46 | 5.51±1.46* | 13.07±1.08* | -17.4±1.11* |
| $Li^{+}$ (n=10) | -32.5±2.29 | 5.51±0.29 | 1.4±2.36 | 2.33±0.32* | 12.5±1.18* | -28.7±3.35 |

*; Significant when compared to control ($p<0.05$).

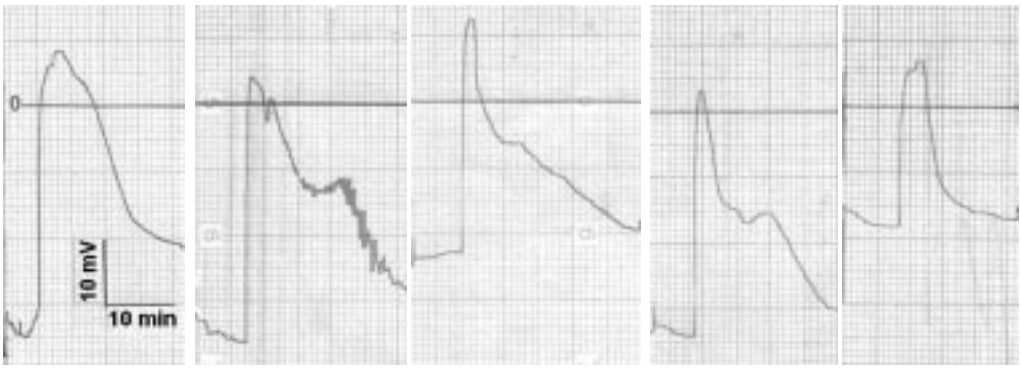


Figure 2. Illustration of records taken from the control (A), $Cd^{2+}$ (B), $Zn^{2+}$ (C), $Li^{+}$ (D), and $Al^{3+}$ (E) groups.

$Cd^{2+}$ (Table, Fig. 3A), and was nonsignificantly more positive with $Al^{3+}$ and less positive with $Li^{+}$ ($p>0.05$; Table, Fig. 4A-B).

**Duration of Effective Fertilization Potential ($EFP_d$):** $EFP_d$ value recorded in the control group was 10.25±1.07 min. $Al^{3+}$ and $Li^{+}$ shortened this duration significantly (5.51±1.46; 2.33±0.32 min respectively; $p<0.05$) (Table, Fig. 4A-B). It did not differ significantly in the groups exposed to $Zn^{2+}$ or $Cd^{2+}$ ($p>0.05$, Table, Fig. 3A-B).

**Duration of Fertilization Potential ($FP_d$):** $FP_d$ value recorded in the control group was 17.0±1.36 min. It was found to be significantly prolonged by $Cd^{2+}$, and significantly shortened by $Al^{3+}$ and $Li^{+}$ (25.49±1.29, 13.07±1.08, and 12.5±1.18 min, respectively; $p<0.05$; Table, Fig. 3A and 4A-B). In the group exposed to $Zn^{2+}$, it resembled that of the control value (Table, Fig. 3B).

**Membrane Potential of Fertilized Egg ($MP_f$):** The mean $MP_f$ value, which was found to be –25.42±0.86 mV in the control group, was significantly less negative when $Al^{3+}$ was added to the solution (-17.4±1.11 mV; $p<0.05$; Table, Fig. 4B). It did not differ significantly in the other experimental groups (Table, Fig. 3A-B and 4A).

## Discussion

Findings of the present study prove that accumulation of $Li^{+}$ and $Al^{3+}$ in the external environment can significantly affect bioelectrical potential parameters of the frog egg which are important for its fertilization, and thus can prevent the effective block against polyspermy; in contrast, $Cd^{2+}$ and $Zn^{2+}$ concentrations used in this study do not influence the above parameters in an apparently negative manner.

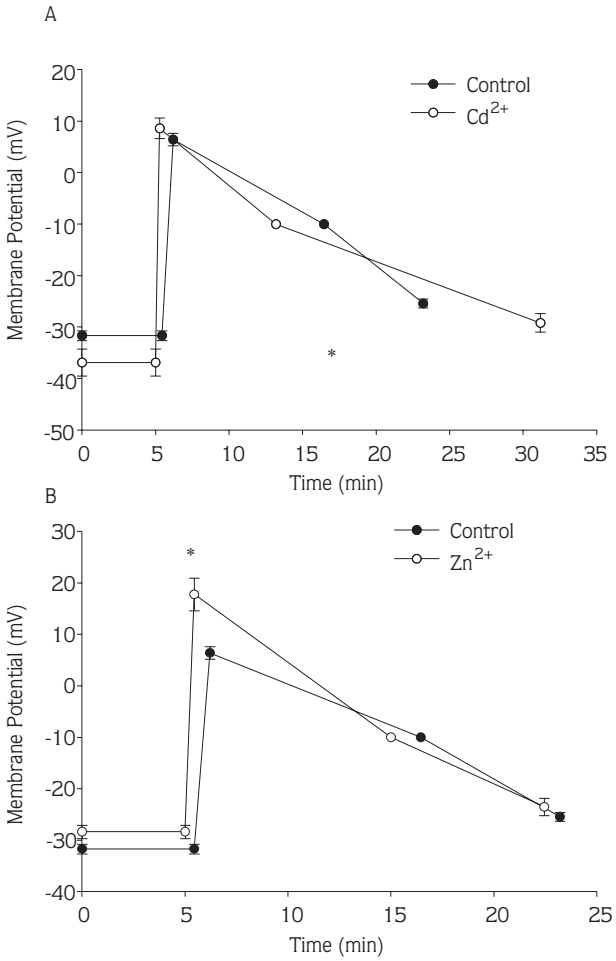


Figure 3. Comparison of values (Mean ± SEM) determined in the control – $Cd^{2+}$ (A), and control-$Zn^{2+}$ (B) groups. $Cd^{2+}$ prolongs duration of fertilization potential significantly, and does not exert any significant effect on the other parameters. In the $Zn^{2+}$ group, peak fertilization potential is significantly more positive, and the other parameter resembles that of the control (*: significant when compared to the control, $p<0.05$).

**Effects of Lithium:** Lithium did not significantly affect the potential parameters recorded before activation (RMP and $F_t$) or after the activation is completed ($MP_f$) (Table, Fig. 4A). However the properties of potential change induced by activation ($FP_p$, $EFP_d$, $FP_d$) were evidently influenced by exposure to $Li^+$ (Table). When activated by a sperm, calcium is released from intracellular depots, and this triggers the transient electrical block (FP) and the following cortical granule exocytosis which establishes the permanent block against polyspermy

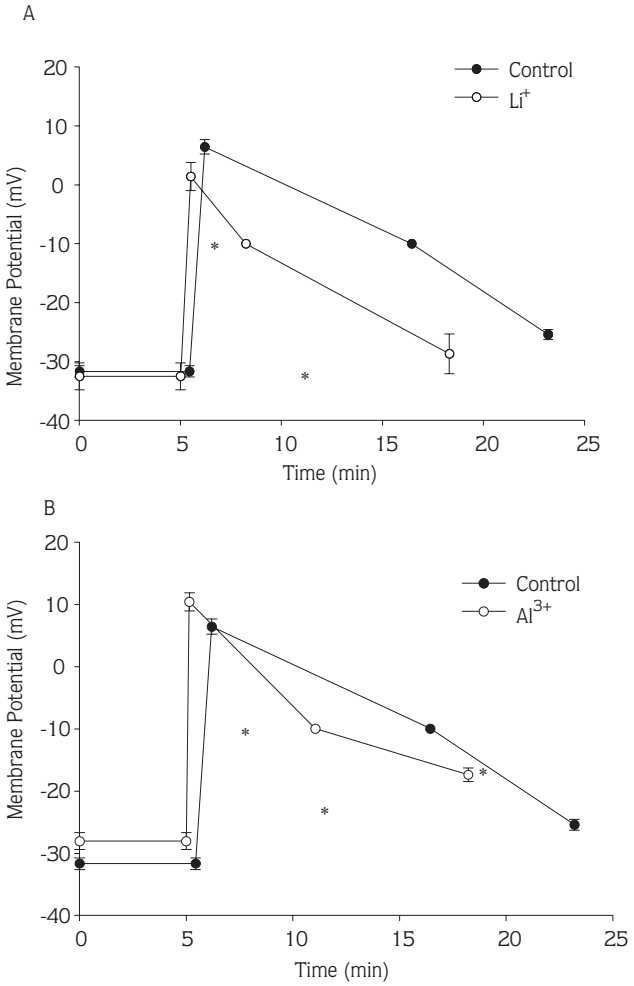


Figure 4. Comparison of values (Mean ± SEM) determined in the control – $Li^{+}$ (A), and control-$Al^{3+}$ (B) groups. $Li^{+}$ and $Al^{3+}$ significantly shortened $EFP_d$ and $FP_d$. $Al^{3+}$ exposure also resulted in more positive $MP_f$ value (*: significant when compared to the control, $p<0.05$).

(4,24,33). The molecular events leading to ooplasmic $Ca^{2+}$ increase have not been clearly defined yet, but mechanisms involving cyclic ADP ribose (34), $IP_3$ (34,35,36), ryanodine receptors (37), cGMP (38) and CICR (39) have been reported. Lithium has been shown to block $IP_3$ cycle by inhibiting inositol-1-phosphatase (25,26); this may lead to limitation of ooplasmic $Ca^{2+}$ increase triggered by fertilization, which may in turn result in inadequate increase of $Cl^{-}$ conductance induced by calcium. In brief, lithium exposure may be the cause of nonsignificantly

decreased $FP_p$ value (Table, Fig.4A) by interfering with the $IP_3$ cycle. When the depolarizing force leading to genesis of FP (increased $Cl^-$ conductance) is diminished, repolarizing force (increased $K^+$ conductance) will be effective much sooner (3). In this group repolarization begins as soon as the peak FP is reached and there is no delay in repolarization resembling a plateau which is observed in the control group at the level of peak FP value (Fig.2D). These may explain the significant decreases in $EFP_d$ and $FP_d$ determined in the present study. It has been reported that the effective electrical block in the frog egg is established at membrane potential values less than –10 mV, and that mechanical block is established at least 2 minutes after genesis of FP (6,7); so it may be concluded that the minimum essential value for $EFP_d$ has to be 2 minutes. In the $Li^+$ group, slow genesis of depolarization induced by fertilization (in 1-2 min), and establishment of effective membrane potential in a much shorter period during the decay phase of FP (2.33±0.32 min; Table, Fig.2D and 4A) might lead to second sperm entry, and thus increase the risk of polyspermy.

**Effects of Aluminium:** As also determined in the $Li^+$ group, $Al^{3+}$ did not significantly influence resting membrane potential and fertilization time in the frog egg, but did affect durations of potential change induced by fertilization ($EFP_d$ and $FP_d$) significantly. Cellular toxic effects of aluminium have already been determined in a number of studies (19,40,41,42). $Al^{3+}$ has been reported to exert toxic effects by interacting with $Ca^{2+}$-binding sites in the rat liver, kidney and brain tissues in vivo (43); in another study, it was shown to block the second messenger system by affecting the GTPase cycle (42). $Al^{3+}$ may thus limit the binding sites of ooplasmic calcium that are increased by activation and/or may decrease the duration of potential change triggered by calcium via its inhibitory effects on cGMP. Aluminium did not influence $EFP_d$ as evidently as $Li^+$ did; however acidic shifts in the environmental pH have been shown to potentiate $Al^{3+}$ toxicity (27) and acidity alone is enough to exert deleterious effects on the fertilization potential (44). Therefore accumulation of $H^+$ and $Al^{3+}$ as a fact of industrial pollution may interfere with fertilization in externally fertilizing species such as frogs by potentiating each other's deleterious effects.

Membrane potential of the fertilized frog egg ($MP_f$) is affected by $K^+$ conductance (3,45). Significantly less negative $MP_f$ values determined in the present study may thus be due to the effect of $Al^{3+}$ on $K^+$ conductance, the mechanism of which could not be clearly explained.

**Effects of Cadmium:** Cadmium significantly prolonged the duration of fertilization potential ($FP_d$), but did not exert prominent effects on the membrane potential values. It has been reported that cadmium competes for divalent cationic binding sites, and this effect is antagonized by divalent metals such as $Mg^{2+}$ and $Zn^{2+}$ (19,20). The results of our study may reveal that $Cd^{2+}$ does not interact during the release of $Ca^{2+}$ from intracellular depots (it did not vary $FP_p$ value significantly), but can influence the transport of ooplasmic $Ca^{2+}$ back into the cellular depots or out of the frog egg. Competition of $Cd^{2+}$ for the binding sites of $Mg^{2+}$ during active transport of ooplasmic $Ca^{2+}$ back to the intracellular depots by $Mg^{2+}$-$Ca^{2+}$ ATPase, and/or interaction of $Cd^{2+}$ with $Ca^{2+}$ during transport of this ion out of the cell by the aid of $Ca^{2+}$ pump

and $Na^{+}$-$Ca^{2+}$ exchange system (46), may explain the significant prolongation of $FP_d$ determined in this study. The cadmium dose used in our study did not influence the potential change triggered by fertilization to the extent of increasing polyspermy risk, and can thus be concluded to be nontoxic; however embryotoxic and teratogenic effects of cadmium are well known on different types of species (19,21,22,32,47).

**Effects of Zinc:** Records obtained from the $Zn^{2+}$ group were similar to those from the control group; however the $FP_p$ value was found to be significantly more positive, and the appearance of recorded FP differed from that of the control (plateau was recorded at nearly –6 mV; Fig. 2C). It has been reported that zinc binds to and antagonizes the effects of powerful chelating agents such as EDTA or less powerful ones such as trisodium citrate and trisodium nitrilotriacetate (19). Trisodium citrate and trisodium nitrilotriacetate are known to act by diminishing cadmium and mercury toxicities in the rat, in relation to cellular defence mechanisms (According to Kurzel (19), from: Schapf et al. Nature 239:231,1972, and Nolen et al. Toxicol Appl Pharmacol 23:238,1972). These weak chelating agents can also bind calcium, and the rise in ooplasmic calcium when they are antagonized by zinc may explain the more positive $FP_p$ values determined in the $Zn^{2+}$ group. The rapid initial repolarization recorded in this group showed a plateau at nearly –6 mV, resulting in a decrease of the repolarization rate; this observation may reveal that $Zn^{2+}$ binds to these chelating agents in a weak manner and for a short period. Studied zinc concentrations did not show deleterious effects on the magnitude and duration of bioelectrical potentials triggered by fertilization and it may thus be concluded that zinc accumulation does not interfere with successful fertilization in externally fertilizing species. However there are some studies reporting that this cation exerts dose-dependent embryotoxic and teratogenic effects during embryonic development (19,20).

## Conclusion

The metals that were previously reported to have embryotoxic/teratogenic effects may significantly influence the genesis of bioelectrical potentials which are important in the frog egg which is fertilized externally. $Li^{+}$ and $Al^{3+}$ may especially increase the risk of polyspermy via their deleterious effects on these potentials, whereas concentrations of $Cd^{2+}$ and $Zn^{2+}$ used in this study do not interfere with the fertilization potential of the frog egg.

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