Weak Magnetic Field at 16 Hz Affects Cardiac Myocyte Ca^{2+} Transients and Reduces Cells Damage Caused by Hypoxia

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Abstract: Effects of extremely low intensity and frequencies of Weak Electromagnetic Fields (WMF) on the cardiovascular system have been reported. However, little is known about the physical mechanism of interactions between magnetic fields and physiology. The aim of this study was to explore the effect of WMF at Extremely Low Frequencies (ELF) on Ca^{2+} transients, and to investigate the influence of magnetic field following hypoxic stress, in cardiac cell cultures. Indo-1 loaded cells, which exposed to WMF at 16 Hz, demonstrated a faster reduction in cytosolic Ca^{2+} transients compared to control cells (75±4% *vs.* 25±8%). This effect was not observed in other low frequencies. BDM (2,3-butanedione 2-monoxime), which inhibits contraction but not Ca^{2+} transient, did not inhibit the effect of WMF on the reduction of Ca^{2+} transients at 16 Hz, suggesting that the effect probably involves ion regulation. However, treatment with the K_{ATP} channel blocker, glibenclamide, followed by 16 Hz exposure, blocked the reduction in cytosolic Ca^{2+} transients, while treatment with chromanol 293B, a selective blocker of the delayed rectifier K^+ current channels (I_{ks}), or the potassium channel opener, pinacidil, did not inhibit the effect. In addition, cardiomyocytes exposure to WMF-ELF at 16 Hz 30 min prior to hypoxia, exhibited a significant reduction in lactate dehydrogenate level and low percentages of death cells *vs.* hypoxic treatment. In conclusion, WMF at low frequency of 16 Hz induced changes in cardiomyocyte contractions by reducing Ca^{2+} transients, most probably through activation of K_{ATP} ion channels. Cells subjected to WMF frequency prior to hypoxic stress, exhibited significantly reduced cardiomyocytes damage.

Keywords: Extremely low frequencies, weak magnetic field, Ca^{2+} transients, Cardiac myocyte, hypoxia.

INTRODUCTION

All life on earth is bathed in a sea of natural- low frequencies and low intensities electromagnetic fields. These fields exhibit extremely low frequencies (ELF) spectrum, which is below 300 Hz, and very low intensity in the range of few picotesla (pT) up to nanotesla (nT), with the earth's geomagnetic field typically around 40 microtesla (μT). Electrical currents exist in the body that is capable of producing magnetic field that extends outside the body [1]. Consequently, they can be influenced by external electromagnetic field as well. Excitable cells, such as nerve and muscle cells (including heart muscle cells), can be directly stimulated by induced currents of sufficient intensity [2]. Intensities below those required for stimulation, might affect electrochemical processes at the cellular level, and may be responsible for alterations in ion transport across membranes, voltage sensitive or other channels, membrane protein function, or binding of hormones or mitogens at the cell surface [2]. Effects of ELF– Weak Electromagnetic Fields (WMF) on different cells were reported [3,4]. Initial studies imposed WMF in the nervous system, centered on modulation of brain ionic mechanism [5-12]. It was shown that application of 16 Hz low amplitude WMF affected $^{45}Ca^{2+}$ efflux from chicken brain slices [6-9], and that WMF of 80 nT at 15.95-16.00 Hz induces cardiac protection following experimental long term coronary artery occlusion [13]. In the cardiovascular system, changes in electrocardiograms of humans, pigs, and rats after exposure to ELF-WMF, have been communicated [14]. A significant increase in systolic blood pressure during field exposure, but no effects on heart rate during the same period, was reported [15]. Other studies suggested that application of 50 Hz field with magnetic flux densities between 1.02 and 15.43 μT, on 26 male volunteer's, decreased heart rate [16]. In addition, it was shown that low frequency magnetic fields induce rapid transitory intracellular expression of heat-shock proteins that mediate a wide range of cellular stress responses including oxygen stress in cardiac cells [17-19]. Following heart anoxia in chick embryo, an increased survival rate of 68.7% was reported, when exposed to ELF of 60 Hz and 4-10 μT [20]. Yet, little is known about the physical mechanism of interactions between magnetic fields and physiology. The purpose of this study was to determine the effect of ELF-WMF on isolated cardiac myocytes contractions and to explore the involvement of potassium channels in the WMF

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effect. In addition, we investigated the influence and the capability of pretreatment with ELF-WMF to protect cardiac cells from hypoxic stress.

METHODS AND MYTHOLOGY

Cell Culture

Rat hearts (2–3 days old) were removed under sterile conditions and washed three times in phosphate buffered saline (PBS) to remove excess blood cells. The hearts were minced and then gently agitated in RDB, a solution of proteolytic enzymes, prepared from fig tree extract (Biological Institute, Ness-Ziona, Israel). RDB was diluted 1:100 in Ca^{2+}- and Mg^{2+}-free PBS at 25°C for a few cycles of 10 min each, as previously described [21,22]. Dulbecco's modified Eagle's medium, supplemented with inactivated 10% horse serum (Biological Industries, Beit Haemek, Israel) and 0.5% chick embryo extract, were added to supernatant suspensions containing dissociated cells. The mixture was centrifuged at 300 g for 5 min. The supernatant phase was discarded, and the cells were resuspended in the same medium. The suspension of the cells was diluted to 1.0×10^6 cells/ml, and 1.5 ml of the suspension was placed in 35-mm collagen/gelatin-coated plastic culture dishes or cover glasses. The cultures were incubated in a humidified atmosphere of 5% CO_2, 95% air at 37°C. Confluent mono-layer exhibiting spontaneous contractions were developed in culture within 1-2 days.

Intracellular Ca^{2+} Measurements

Intracellular free calcium ($[Ca^{2+}]_i$) from individual cardiomyocytes was measured using the indicator indo-1-AM (Molecular probes, Oragon, USA) under a Zeiss epi-fluorescent inverted microscope. The ratiometric methods for quantification of the results have been described pre-viously [23]. Cardiomyocytes grown on a cover glass were incubated with 2 µM indo-1-AM and 1.5 µM pluronic acid for 60 min in glucose-enriched PBS at 25 ^{0}C. After incuba-tion, the cells were rinsed twice with glucose-enriched PBS and transferred to a chamber on the microscope. Indo-1 loaded cells were excited at 340 nm and the emitted light then split by a dichroic mirror into two photomultipliers (Hamamatsu, Japan), with input filters at 410 and 490 nm for indo-1. The fluorescence ratio of 410/490 nm, which is proportional to $[Ca^{2+}]_i$, was implemented to the Ca-plan program [23].

Application of Magnetic Field

A digital waveform was connected to a copper wire coil with a single wrapping of diameter 3.5 cm and alternating sinusoidal currents in range of 1-100 KHz with amplitudes of few mA. While applying 500 mV we generated magnetic fields with different amplitudes in range of few pT up to 100 nT, according to the Biot-Savart law. In the current report sinusoidal currents of 15 Hz, 16 Hz, or 17 Hz were applied. In all experiments we measured that the geomagnetic intensity field in the plain of the cells was 40 µT. The copper wire was wound around the dish with the cardiac myocytes cultures.

Drugs Application

BDM (2,3-butanedione, 2-monoxime) (10 mM), potassium channel opener, pinacidil (3 µM), potassium channel blockers, glibenclamide or chromanol 293B (5, 2 µM respectively), were added to cells medium for 15 min. Then, a magnetic field at 16 Hz frequency was applied to cells for farther 30 min. The changes in $[Ca^{2+}]_i$ were monitored each 5 min. In several experiments, the magnetic field was turned off after 30 min of exposure, and $[Ca^{2+}]_i$ recording continued in order to observe recovery of Ca^{2+} transients.

All chemicals were obtained from Sigma (St. Louis, USA), unless other is mentioned.

Hypoxic Condition

Cardiomyocyte cultures, 5- to 7-days old, were washed from the medium with glucose-free phosphate-buffered saline containing 10 mM HEPES at pH 7.4 before exposing the cells to the hypoxic conditions at 37°C. The hypoxic condition consisted of 120 min in a hypoxic incubator where the atmosphere was replaced by the inert gas argon (100%) in Glucose-free media [21, 22]. The hypoxic damage was characterized at the end of the hypoxic period by propidum iodide (PI) staining and release of lactate dehydrogenate (LDH) to the cell medium.

Release of LDH

Protein content and LDH activity were determined according to Yitzhaki *et al.* [22]. Briefly, 25 µl supernatant was transferred into a 96-well dish, and the LDH activities were determined with an LDH-L kit (Sigma), as described by the manufacturer. The product of the enzyme was measured spectrometrically at 30˚C at a wavelength of 340 nm as described previously [22]. The results were expressed relative to the control (X-fold) in the same experiment. Each experiment was done in triplicate and was repeated at least three times.

Propidium Iodide Staining

The assay is based on vital binding of PI (5 µg/ml) to nuclei of cells whose plasma membranes have become permeable because of cell damage. The assay was performed according to Leshem-Lev *et al.* [24].

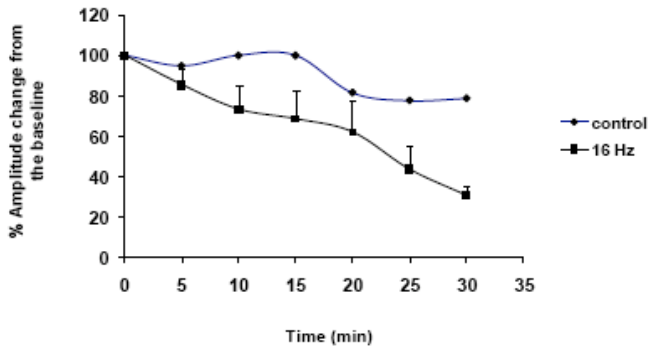
Data Analysis

Parameters were normalized to 100 percent and all values are presented as percent change from baseline. Mean and SD were calculated and a $p\leq0.05$ was considered statistically significant.

RESULTS

Isolated rat cardiac myocyte contractions, were subjected to WMF at intensity of 40 nT at 16 Hz. After 30 min expo-sure, a reduction in the amplitude of cytosolic Ca^{2+} transients relative to initial transient, was demonstrated (75±4% *vs.* control 25±8%, Fig. **1A**). This effect was time dependant

(A)



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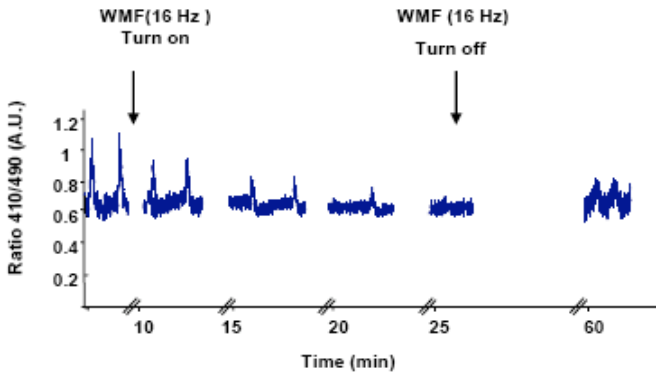

Fig. (1). Cardiac cells, 4 days in culture, were subjected to magnetic field at frequency of 16 Hz, and calcium transients were recorded using the fluorescent probe Indo-1. A reduction of in the amplitude of cytosolic Ca^{2+} transients in the 16 Hz treated cells *vs.* control relative to initial transient was demonstrated. Results are presented as percentages change from the initial Ca^{2+} transient amplitude (**A**). 30 min after exposure, the magnetic field was turned off, and calcium transient was recorded. Recovery of the cells was observed, indicating that the WMF effect on cells is reversible (**B**). Recording were preformed each 5 min for 30 sec, for total 30 min stimulation. First 10 sec are presented. Data are means of at least seven replicates $P < 0.001$.

and reversible, meaning that amplitude of Ca^{2+} transients were reappeared approximately 30 min after stopping the magnetic field exposure (Fig. **1B**). In order to investigate the effect of other WMF frequencies on cardiomyocytes, cells were treated with WMF at 15 Hz or 17 Hz. In the 15 Hz treated cells, no reduction in the amplitude of Ca^{2+} transients was observed. Moreover, a small elevation in Ca^{2+} amplitude relative to initial transient *vs.* control cells was demonstrated (23±5% *vs.* 30±6%, Fig. **2**). However, no significant change in the Ca^{2+} amplitude *vs.* control cells was observed when cardiac cells were subjected to stimulation of 17 Hz.

In order to determine if the magnetic field affects the mechanical actin-myosin contraction or induces changes in

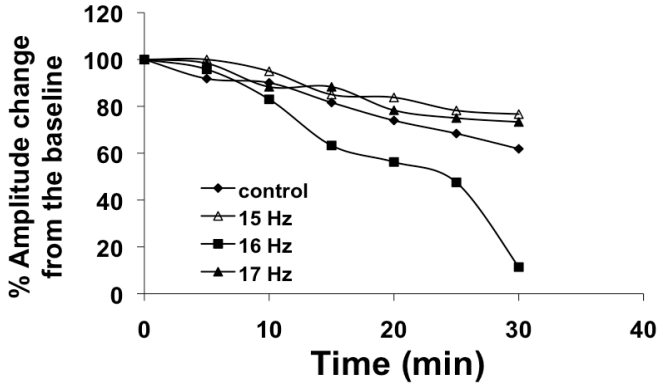

Fig. (2). Cardiac cells, 4 days in culture, were subjected to magnetic field at frequencies of 15 Hz, 16 Hz or 17 Hz. Intracellular calcium transient was recorded using the fluorescent probe Indo-1. A small elevation in Ca^{2+} amplitude relative to initial transient in 15 Hz treated cells *vs.* control cells was demonstrated. However, no significant change in the Ca^{2+} amplitude was observed when cardiac cells were subjected to 17 Hz. Changes in the amplitude of Ca^{2+} transient were recorded each 5 min for 30 sec, for a total 30 min stimulation. Results are presented as percentages change from the initial Ca^{2+} transient amplitude. Data are means of at least five replicates $P < 0.01$.

ion homeostasis, we treated the cells with BDM, which inhibits myosin activity but not Ca^{2+} transients. Stopping of cardiomyocyte contractions was observed after treatment with BDM (10 mM), while no effect on Ca^{2+} amplitude *vs.* control cells was demonstrated (25±1% *vs.* 25.3±3% Fig. **3**). Application of BDM before 16 Hz, did not abolished the reduction in the amplitude of Ca^{2+} transient, which was decreased to 63±4% from the initial transient (Fig. **3**), suggesting that the effect of the magnetic field on intracellular Ca^{2+} is not via the actin–myosin mechanism.

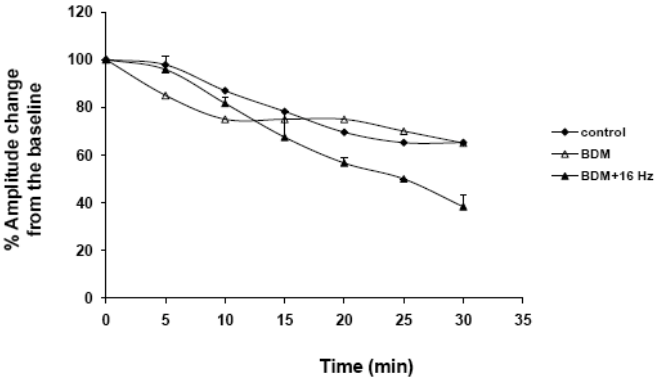

Fig. (3). Indo-1–loaded cardiomyocytes were pretreated with 10 mM BDM, an inhibitor of myosin-actin contractions, for 15 min before application of magnetic field at frequency of 16 Hz for further 30 min. As revealed, application of BDM before 16 Hz did not abolish the reduction in Ca^{2+} transient amplitude. The fluorescence ratio of 410/490 nm, which is proportional to changes in Ca^{2+} transients, is demonstrated. One experiment shown is representative of three.

It was found that the resonant frequency for potassium in the earth's magnetic field is close to 16 Hz, and exposure to the latest can activate the ATP dependent potassium ion channels, present in myocardial cell membrane [26]. Thus,

we conducted experiments in order to explore the involvement of potassium channels in the WMF. Cardiomyocytes were subjected to the selective K_{ATP} channel opener, pinacidil. A decrease of 32 ± 4% in the amplitude of cytosolic Ca^{2+} transients *vs.* control was observed, 10 min after pinacidil application. An additional reduction of 20 ± 2% was observed following a magnetic field exposure (Fig. **4**). However, treatment with K_{ATP} channel blocker, glibenclamide,

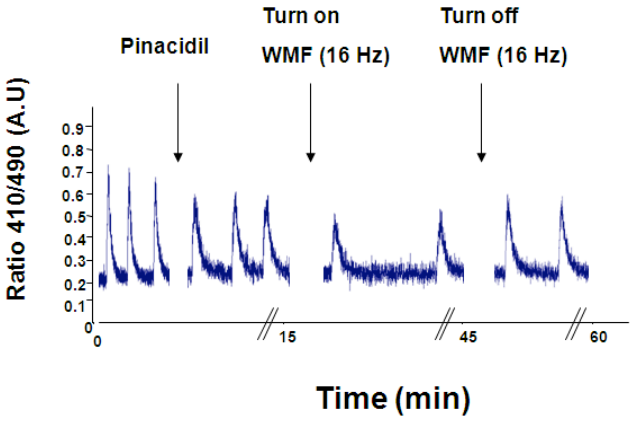

Fig. (4). Indo-1–loaded cardiomyocytes were pretreated with 3 μM pinacidil, a potassium channel opener, for 15 min before application of magnetic field at frequency of 16 Hz for further 30 min. Adecreased amplitude of cytosolic Ca^{2+} transients *vs.* control was observed, 10 min after pinacidil application, followed by an additional reduction after magnetic field exposure. The fluorescence ratio of 410/490 nm is demonstrated. One experiment shown is representative of three.

followed by WMF at 16 Hz, abolished the reduction in the amplitude of cytosolic calcium transients induced by the magnetic field. Moreover, an elevation of 10±8% from the initial Ca^{2+} amplitude compared to reduction of 25±3% in control cells, was observed (Fig. **5**). No significant inhibiting

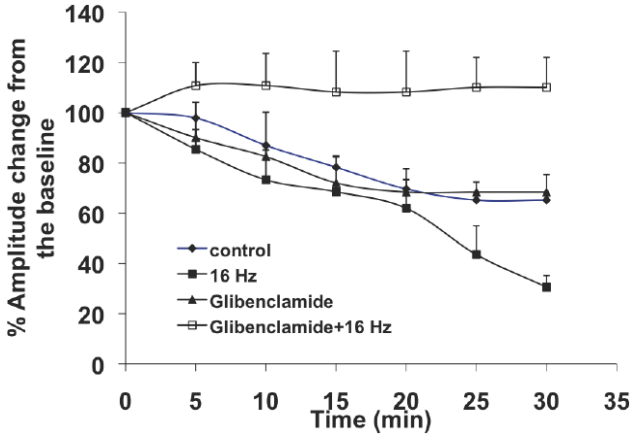

Fig. (5). Indo-1–loaded cardiomyocytes were pretreated with 5 μM glibenclamide, a K_{ATP} channel blocker, for 15 min before application of magnetic field at frequency of 16 Hz for further 30 min. Treatment with glibenclamide, followed by WMF at 16 Hz, abolished the reduction in the amplitude of cytosolic calcium transients induced by the magnetic field. The fluorescence ratio of 410/490 nm, is demonstrated. Data are means of at least 4 replicates ± SD. $P<0.001$.

effect was observed when cells were exposed to the selective blocker of the delayed rectifier K^+ current (I_{Ks}), chromanol 293B, followed by magnetic field (Fig. **6**). These results suggest that the 16 Hz treatment effect on cardiomyocytes is probably mediated *via* the K_{ATP} channels and not through I_{Ks} voltage-gated channels.

The fact that ATP sensitive potassium channels are mediated in ischemic preconditioning (IPC) protection [27], leaded us to farther investigate the effect of magnetic field at 16 Hz on cardiomyocytes protection from hypoxic damage. Cells were exposed to WMF for 30 min prior to subjection to hypoxic stress. No difference on LDH release or cell death was observed in control cells when compared to cells exposed to magnetic field treatment. However, a significantly reduced LDH level in the medium were obtained in pretreated cells, with WMF and then subjected to hypoxia, compared to cardiomyocytes which were subjected to hypoxia only (0.28±0.02% *vs.* 0.59±0.03%, $p<0.01$). In addition, cells which were expose to WMF, exhibited lower percentage of cell death as revealed from PI staining (1.91±0.4% *vs.* 4.1±0.1%, $p<0.01$) (Fig. **7**). Similar results were obtained when cardiomyocytes were subjected to WMF at 16 Hz for 30 min, 2 hr before subjected to hypoxic condition, indicating the persisting of the cardioprotection (Fig. **8**).

DISCUSSION

In this study we showed that short exposure to WMF at frequency of 16 Hz, affects cardiac myocyte contractions by significantly reducing the amplitude of Ca^{2+} transient. The presented results indicate that the reduction in Ca^{2+} transient is not mediate via the actin – myosin mechanism, since treatment with BDM, an inhibitor of cell contractions, did not inhibit Ca^{2+} transient and the decreasing in the levels of the amplitude of cytoplasmic Ca^{2+}, following exposure to WMF. Therefore, we assume that the effect of the magnetic field on cell contractions is probably occurs through changes in intracellular ions regulation.

One of our main finding in this work suggests that the effect of WMF on cardiomyocytes was exclusively observed after application of magnetic field at 16 Hz frequency and not at 15 Hz or 17 Hz frequencies. These results join to other experimental evidences of biological effects of ELF-WMF, which supported by theoretical models involving quantum-interference effects on protein-bound substrate ion. This ion interference mechanism predicts specific magnetic field frequency and amplitude "window" within which the biological effect would occur [28, 29]. In addition, an idea developed by Liboff *et al.* suggests that the frequency windows for the biological effects of electromagnetic fields were in some way due to ion cyclotron resonance [26]. It was found that the resonant frequency for potassium in the earth's magnetic field is close to 16 Hz, and exposure to the latest can activate the ATP dependent potassium ion channels, present in myocardial cell membrane. To verify the involvements of potassium channels on the WMF effect, experiments with potassium channel opener, and specific potassium channels blockers, were preformed. The significant reduction of 75±4% in Ca^{2+} ion transient that was evident after WMF exposure was arrested by adding glibenclamide, a known K_{TAP} blocker to the culture. However,

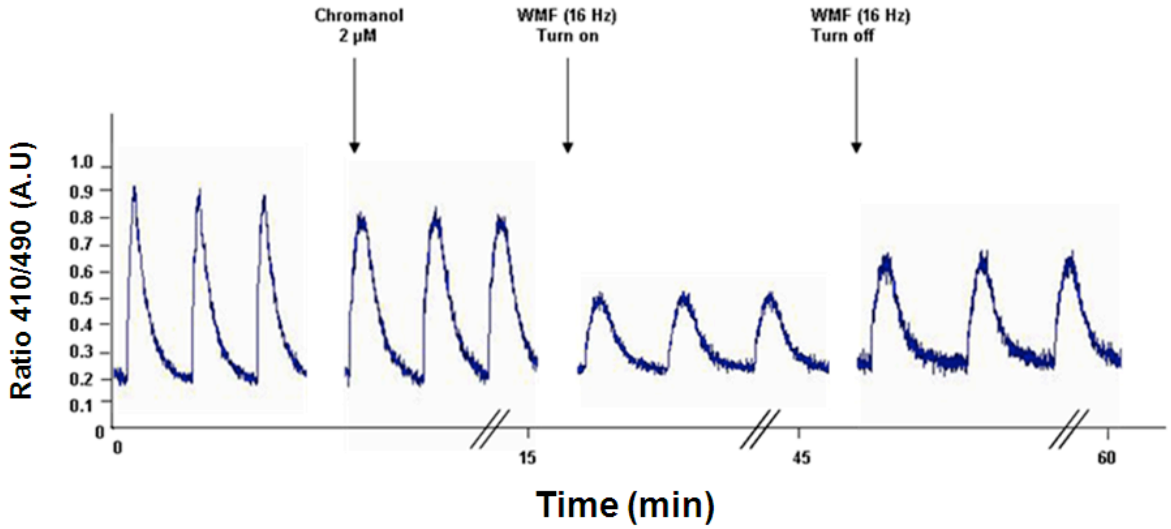

Fig. (6). Indo-1–loaded cardiomyocytes were pretreated with 2 µM Chromanol 293B, a selective blocker of delayed rectifier K^+ current, for 15 min before application of magnetic field at frequency of 16 Hz for further 30 min. No significant inhibiting of the reduction in the Ca^{2+} amplitude of was observed when cells were exposed to chromanol 293B and magnetic field. The fluorescence ratio of 410/490 nm is demonstrated. One experiment shown is representative of three.

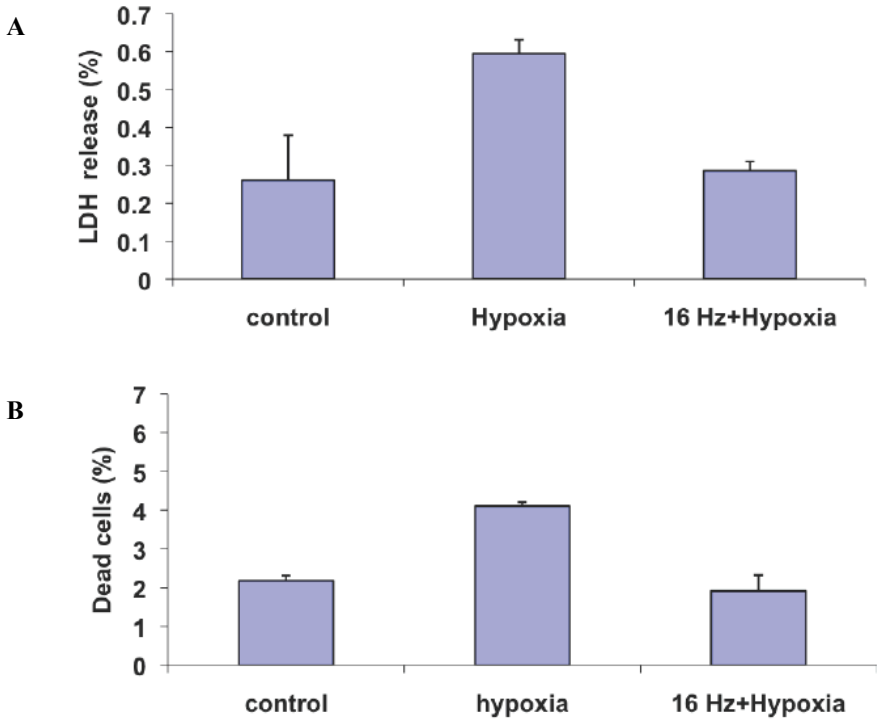

Fig. (7). Six-day-old cardiomyocytes were treated for 30 min with magnetic field at frequency of 16 Hz. The cells were then washed and subjected to hypoxia for 2 h in glucose-free PBS, at 37°C. The amount of LDH released to the medium was determined and compared to the total activity of control homogenate (100%, A). Immediately after hypoxia, the cells were stained with propidium iodide (red), which marks damaged cells (B). A significantly reduced LDH level and dead cells were obtained in pretreated cells with WMF at 16 Hz and hypoxia, compared to cardiomyocytes which were subjected to hypoxia only. The percentage of dead cells was determined and compared to total number of cells (100%) at the same dish. Data are means of at least three replicates in four separate experiments ± SD. $P < 0.05$.

reduction in Ca^{2+} ion transient was observed when pinacidil (K_{TAP} channel opener) or chromanol 293B (I_{Ks} blocker) was added. These results suggest that the effect of counteracting calcium efflux from the myocytes in culture by glibenclamide preformed by activation the K_{ATP} channels. The latest activation induces closure of sarcollemmal voltage-gated L-type calcium channels as a result of membrane hyperpolarization and a greatly diminished intracellular calcium concentration. Our observation supported by Shon *et al.*, which showed that following exposure of WMF, increased potassium currents at cell membrane channels were occurred [14]. Therefore, we can speculate that the observed changes

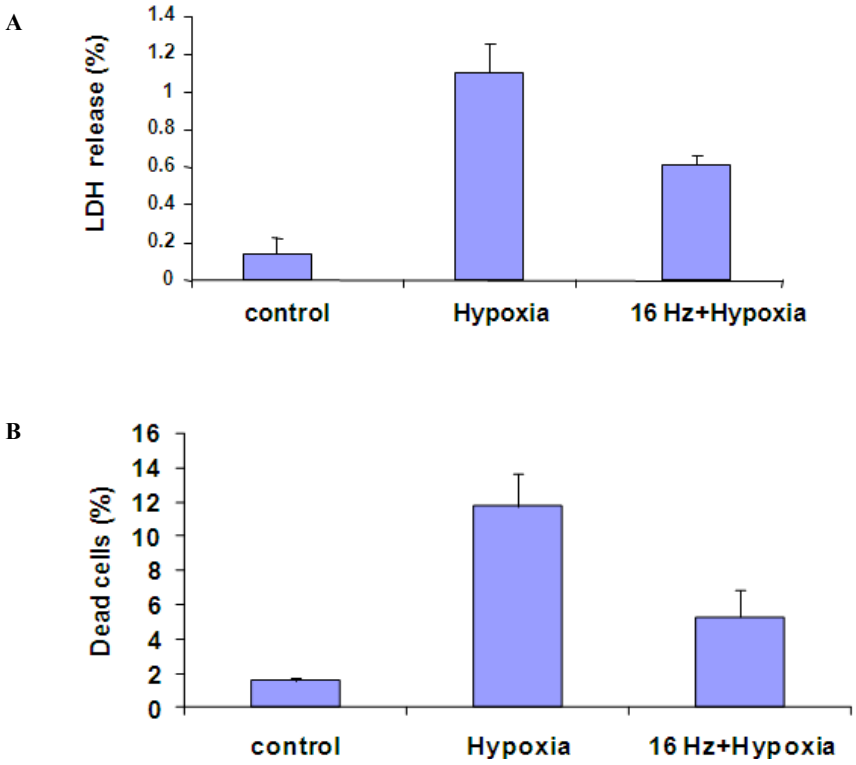

Fig. (8). Six-day-old cardiomyocytes were treated for 30 min with magnetic field at frequency of 16 Hz. After 2 hr, the cells were washed and subjected to hypoxia for 2 h in glucose-free PBS, at 37°C. The amount of LDH released to the medium was determined and compared to the total activity of control homogenate (100%, A). Immediately after hypoxia, the cells were stained with propidium iodide (red), which marks damaged cells (B). A significantly reduced LDH level and dead cells were obtained in pretreated cells with WMF at 16 Hz 2 hr before subjection to hypoxia, indicating the persistence of IPC effect mediated by a WMF exposure. Percentage of dead cells was determined and compared to total death amount (100%) at the same dish. Data are means of at least three replicates in four separate experiments ± SD. $P < 0.05$.

are most probably due to activation of cardiac K_{ATP} membrane channels, and not the I_{Ks} voltage-gated channels; however we cannot exclude involvement of other ion channels such as Na^+ or Ca^{2+} ion channels in the WMF effect.

Myocardial infarction and heart failure are leading causes of morbidity and mortality in humans. Considerable effort has been devoted to improving functional recovery and reducing the extent of infarction after ischemic episodes. A step in this direction was the discovery that the heart is significantly protected against ischemic injury when first preconditioned by a brief ischemia [30]. Other studies showed that this phenomenon, termed as Ischemic Precondition, can be mimicked by several pharmacological agents [22, 25]. Growing evidences imposed that Protein Kinase C (PKC) activation together with the opening of the K_{ATP} channels, are the main players in the mechanism associated with IPC protection [27]. The presented finding in which the ATP sensitive potassium channels are involved in the WMF effect, and the fact that these channels mediated IPC protection, leaded us to farther investigate the influence and the capability of ELF-WMF to protect the cardiac cells from hypoxic stress. Decreased LDH levels released to the cell's medium as well as reduction in PI staining were observed in cardiac cells that were pretreated with WMF at 16 Hz before subjection to 2 hr of hypoxic condition. Similar results were obtained when exposure to WMF was done 2 hr (Fig. **8**) or 24 hr before hypoxia (data not shown), meaning that WMF treatment elicits an early and late IPC. Jointly with our findings, it has been reported that chick embryos which were subjected to heart anoxia and treatment at ELF-WMF demonstrated increased survival rate [20]. The presented results indicate that WMF have a significant protective impact on the surviving cardiac myocytes. The proposed mechanism involves channel opening which leads to hyperpolarization of the membrane and enhances shortening of the cardiac action potential, by accelerating phase 3 of repolarization. In turn, inhibition of Ca^{2+} ion entry into the cell via L-type channels will occur, and prevention of Ca^{2+} overload will be achieved [27]. Inhibition of Ca^{2+} overload in the cell plays an important role in states of metabolic stress such as cardiac ischemia. The present study showed that WMF at frequency of 16 Hz induces changes in cardiomyocyte contractions by reducing Ca^{2+} transients, most probably through activation of ATP-sensitive potassium channels, and treatment with WMF prior to hypoxic stress, exhibited a significant cardioprotection against hypoxic damage. Yet, additional studies need to be performed to explore the involvement of potassium channels in the WMF induced cardioprotection.

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