



Research Article

In vitro Effect of Radiofrequency on hsp70 Gene Expression and Immune-effector Cells of Birds

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ABSTRACT

Poultry birds under free range system of management are a good biological indicator for low-intensity Electro-Magnetic Frequency radiations (EMF-r) for their vulnerability to EMF-r. The effect of EMF-r on different biological systems was extensively studied but no consistent effects had been inferred in this regard. With the aim of assessing the *in vitro* effect of radio frequency (RF) exposure on cellular stress and on functional activities of immune-effectors cells of poultry, this study was conducted. Two rural breeds [one best adapted exotic breed-Rhode Island Red (RIR) and one indigenous breed-Haringhata Black (HB)] were used. Blood samples, collected from six healthy birds of each species, were used for neutrophil functional assay by nitro blue tetrazolium (NBT) reduction test. Peripheral blood mononuclear cells (PBMC), obtained from blood were used for lymphoproliferation assay by 3-[4, 5-dimethylthiazol-2yl]-2, 5-di phenyltetrazolium bromide (MTT dye) and expression of hsp70 gene by reverse transcription-polymerase chain reaction (RT-PCR) analysis. Prior to the experiment, PBMC were cultured into tissue culture plates and blood for NBT assay was taken in separate micro-centrifuge tubes from the sample of each bird in duplicates under bio-safety cabinet for radiofrequency exposure. The plates and tubes were exposed to 850 and 1200 MHz radiofrequency field from a standard mobile phone separately along with a power analyzer (SAR 1.7 W/Kg) for 5 minutes, 15 minutes, 25 minutes, 40 minutes and 60 minutes. The common trend of the hsp70 gene expression in RIR and HB breed of birds were observed upon 850 RF exposures from 25 minutes onward. The results showed that upon 1200 MHz RF post exposure, hsp70 gene expression occurred at 25 minutes onward in HB breed birds and 60 minutes post exposure in case of RIR bird. A typical trend of initial significant ($P \leq 0.05$) decrease (5 minutes through 15 minutes post exposure) followed by significant ($P \leq 0.05$) increase in lymphoproliferation from 25 minutes to 60 minutes, was observed in both the breeds and magnitude of exposures. All the test groups no significant variation in superoxide production by neutrophil. It may be concluded that, lower radiofrequency (850 MHz) emitted by mobile phone poses more stressful effect than the higher radiofrequency (1200 MHz) on *in vitro* exposed cells in both the breeds as indicated by expression of hsp70 gene and initial suppression of proliferation ability.

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INTRODUCTION

Mobile telephony is one of the fastest growing industries in the world and expected to exceed one billion subscribers in India by 2013 (TRAI, 2012). This may create biological hazards as a major source of non-ionizing form of Electro-Magnetic Frequency radiations (EMF-r). Although the non-ionizing radiations are considered to be less harmful than ionizing radiations having relatively less power, can produce bio-effects and adverse health effects including heat stress upon prolonged exposure at low intensity below existing exposure standards (FCC, 1999; Kohli et al., 2012; Seletun Scientific Statement, 2011). Poultry birds under free range system of management are vulnerable to such EMF-r exposures and also a good biological

indicator for low-intensity EMF-r due to thin skulls and feathers that act as dielectric receptors of microwave radiation (Balmori, 2005). Heat stress and other kind of stressors (anoxia, heavy metal ion and radiation) rapidly induce enhanced synthesis of heat shock proteins (hsp), important for various intra-cellular processes, like protein-protein interactions, including folding and assisting in establishing of proper protein conformation, and prevention of inappropriate protein aggregation (Givisiez et al., 2011). One of the important heat shock proteins, hsp70 families, is a set of highly conserved protein which acts as molecular chaperone by protecting proteins from denaturation including folding, transport and post-translational modification of protein (Al-Katanani and

Hansen, 2002). Peripheral blood mononuclear cells are the major secretor cells of circulating hsp70 (Hunter-Lavin et al., 2004). Earlier studies on expression of hsp and its related genes on thermal stress have been conducted at molecular level in broilers and layers (Gabriel et al., 1996; Yu and Bao, 2008). Further, exposure of radiofrequency (RF) on human cell cultures and peripheral mononuclear cell and neutrophil had been studied (Capri et al., 2004; Kwee et al., 2001). Cleary et al. (1996) investigated the effect of isothermal radiofrequency radiation on human cytolytic T lymphocytes and revealed that exposure to 2450 MHz RIF radiation at specific absorption rates of greater than 25 W/kg induced a consistent, statistically significant reduction in CTTL-2 proliferation, especially at low IL-2 concentrations. Although several workers had been studied the effect of RF on different biological systems, no consistent effects had been inferred in this regard (WHO, 1993). It is apparent that hsp70 being one of the representative stress proteins, might be an indicator of stress analysis in the context of exposure of radiofrequency on poultry birds. However, the expression study of hsp70 on exposure of radiofrequency (RF) emitted by mobile phone and its tower is not documented so far in rural poultry, more susceptible to RF exposure during scavenging and grazing. In this outset, the present study was conducted on two rural poultry breeds for assessing the *in vitro* effect of RF field exposure on hsp70 gene expression and on functional activities of immune-effector cells.

MATERIAL AND METHODS

The study was conducted on two rural poultry breeds, reared under free-range system in West Bengal, India (latitude: 22.5° – 27.2° N and longitude: 86.1° – 89.8° E). One was best-adapted exotic breed (Rhode Island Red) and the other one was indigenous breed (Haringhata Black, native to West Bengal, India) (Pan et al., 2011; SAPPLPP, 2013). Six healthy birds of 22 weeks old hen from each breed were subjected for the study.

Sample Preparation

Blood samples (4 ml) were collected from wing vein of each bird in an EDTA containing vial in morning. Blood (100 µl) was taken in a sterilized microcentrifuge tube for neutrophil functional assay (NFA). Peripheral blood mononuclear cells (PBMC) were separated from the rest amount of each blood sample following standard protocol (Boyum, 1968) with some modification using Histopaque[®] 1.084 (Sigma) in the ratio of 1:1. Then PBMC were dispensed @ 1X10⁶ per well into 96 well flat bottom tissue culture plates (Nunc, Thermo-Scientific, USA) having 100 µl of RPMI growth medium (Sigma) with 10 % fetal calf serum. The PBMC were cultured into tissue culture plates (n= 11) and blood for NBT assay was taken in eleven separate microcentrifuge tubes from the sample of each bird in duplicates under bio-safety cabinet (Esco Micro Pvt. Ltd., Model No. AC₂-4Li) for radiofrequency exposure.

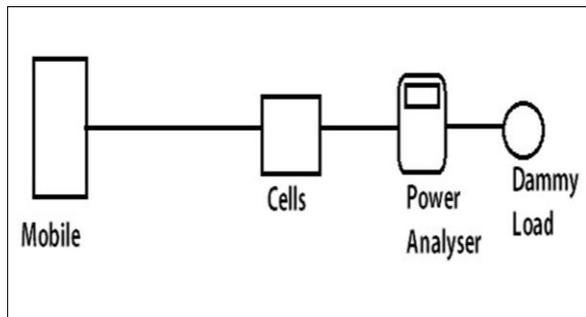


Figure 1: Schematic diagram of Experimental design

Experimental Design and Exposure to Radiofrequency Field
One plate and one tube from the sample of each bird, in duplicate, were exposed to 850 MHz radiofrequency field from a standard mobile phone (Spice, GSM & CDMA Dual Mode, D 88) along with a handheld power analyzer, previously standardized by Radio Test Set (Marconi, Model – 2955) (Figure 1 and Figure 2) (SAR 1.7 W / Kg) for 5 minutes. Similarly, further four plates and tubes of each bird, in duplicate, were exposed for 15 minutes, 25 minutes, 40 minutes and 60 minutes, respectively. In the same way, another five plates and five tubes from the sample of each bird, in duplicate, were exposed to 1200 MHz-radiofrequency source from the same instruments and condition for 5, 15, 25, 40 and 60 minutes, respectively. The rest plate and tube were used as control. The exposed tubes were immediately processed for Neutrophil functional assay (NFA) and the plates were incubated in a CO₂ incubator (New Brunswick, Galaxy 48S) at 37°C with 5% CO₂ tension for further study of stress gene analysis and MTT assay.



Figure 2: Radiofrequency exposure in samples within bio-safety cabinet

Stress Gene Analysis

After 3 hours of incubation, lymphocytes were harvested and washed using centrifugation at 8000 rpm for 8 minutes at 4°C. The cell pellets were washed with DEPC treated water and immediately mRNA was extracted using trizol reagent (Qiagen) and subjected to reverse transcription-polymerase chain reaction (RT-PCR). Initially extracted mRNA was converted to cDNA (Sambrook and Russel, 2001). Specific primers set (hsp-F 5'- GGCTGGTATGCGTGACAT-3' and hsp-R 5'- GGCTGGTATGCGTGACAT-3') were designed from published sequence of avian hsp70 gene (accession no NM_001006685) for amplification of hsp70 from cDNA. The reaction was performed in 50 µl volume containing approximately 75 ng of cDNA, 20 pmol /µl each of forward (hsp-F) and reverse primer (hsp-R), 1.5 mM MgCl₂, 200 µM each dNTPs, 10 X PCR buffer and 2 U of Taq DNA polymerase. PCR was done in a thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany) with initial denaturation at 94°C for 4 minute, followed by 30 cycles with denaturation at 94°C for 1 minute, annealing at 55°C for 50 sec and extension at 72°C for 2 minute. The final extension was done at 72°C for 7 min. The amplified PCR products were separated by agarose gel electrophoresis using 1.5% agarose in 0.5 X TBE along with 0.5 µg/ml ethidium bromide at 100 volt for 1 hour with specific DNA Ladder. The amplified DNA fragments were observed under Gel Documentation System (UVP, UK) and analyzed. The PCR products were subjected for sequencing after excised

from the gel. The nucleotide sequences were aligned and compared using Lasergene software DNA STAR. Phylogenetic comparison was performed using MEGA 4.0.2 software. Searches of homology of genes were performed by BLASTN programme in NCBI website.

Lymphoproliferation Assay (LPA)

After exposure, 100µl of ConA (Sigma, USA) at a concentration of 10 µg/ml was added to 100µl of PBMC suspension into each test well of 96-well tissue culture plate (Nune, Denmark) for non-specific stimulation. Similarly, 100µl of growth medium was added to 100µl of PBMC suspension into each control well. Side by side similar experiments were carried out with the cells in the unexposed (control) plate. The plates were incubated for 48 h at 37°C containing 5% CO₂ tension. The proliferation of PBMC was determined by the colorimetric 3 - [4, 5 - dimethylthiazol-2yl] - 2, 5 - di phenyltetrazolium bromide (MTT) assay (Daly et al., 1995). After 48 h cultures, 20 µl of MTT (Sigma, USA), 5mg/ml in PBS, was added to each well and incubated for 4 h at 37°C. The tissue culture plates were centrifuged at 500 X g for 10 min and then the supernatant was removed carefully without disturbing the cell plates or formazan precipitate. The formazan crystals were dissolved by the addition of 150 µl of dimethylsulphoxide (DMSO, Sigma, USA) followed by 25 µl of glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) and mixed thoroughly with micropipette and incubated at room temperature for 10 min. The formazan development was read at 595 nm using a plate reader (ECIL, India). Lymphocyte proliferation was measured using stimulation index (S.I.) as per the following formula-

$$\text{Stimulation index (S.I.)} = \frac{\text{Mean optical density of test wells having ConA}}{\text{Mean optical density of control wells}} - 1$$

Neutrophil functional assay (NFA)

Radiofrequency exposed blood samples kept in microcentrifuge tubes was used for nitroblue tetrazolium (NBT) reduction test

to assess superoxide production by neutrophils (Siwicki et al., 1985). Briefly, equal volume (100µl) of the blood sample and nitroblue tetrazolium (NBT) solution (0.2% in phosphate buffered saline, PBS) were mixed in the micro-centrifuge tubes and incubated for 30 min in room temperature. Then 50µl cell suspension was added to 1ml N, N dimethylformamide (SRL, India) and centrifuged for 5 min at 3000 g. The optical density (O.D.) of the colour formed was read using spectrophotometer at 540nm.

RESULTS

Stress Gene (*hsp70*) Analysis

RT-PCR yielded an amplification product of 515 bp in positive samples upon 1% agarose gel electrophoresis. Nucleotide sequence of the amplicons of both breeds revealed the product of 515 bp, which was corresponding to the sequence of *hsp70* gene of *Gallus gallus* from 688 bp to 1202 bp (accession no NM_001006685). Nucleotide sequence of *hsp70* gene of HB and RIR breed had 98.8 and 98.4 % identity, respectively. There were changes in nucleotide sequence at 334 and 359 position of *hsp70* gene sequence of RIR breed in compared to HB breed. Variation of expression in *hsp70* gene was observed in poultry birds upon exposure to radio frequency at different time intervals. A general trend of the gene expression in RIR and HB breed of birds was observed upon 850 MHz RF exposures where from 25 minutes onward electrophoresis bands were observed in the gel in both the breeds (Figure 3 and Figure 4). However, 15 minutes post exposure also showed gene expression in HB birds upon 850 MHz RF exposure. The results showed that upon 1200 MHz RF exposure, *hsp70* gene expression took place 25 minutes onward in HB breed birds. However, this only took place on 60 minutes post exposure in case of RIR bird (Table -1).



Figure 3: Analysis of *hsp70* gene expressions in RIR by RT-PCR



Figure 4: Analysis of *hsp70* gene expressions in HB by RT-PCR

Table 1: Expression of mRNA of hsp70 gene in poultry birds after *in vitro* Radio Frequency exposure

Time	RIR bird		HB bird	
	850	1200	850	1200
Control (unexposed)	---	---	---	---
5 min	---	---	---	---
15 min	---	---	+	---
25 min	+	---	+	+
40 min	+	---	+	+
60 min	+	+	+	+

Lymphoproliferation assay (LPA)

The results revealed a typical trend of initial significant ($P \leq 0.05$) decrease (5 minutes through 15 minutes post exposure) followed by significant ($P \leq 0.05$) increase in lymphoproliferation from 25 minutes to 60 minutes post exposure. This general trend was observed in both 850 MHz and 1200 MHz RF exposure and in both the breeds (Table – 2).

Neutrophil Functional Assay (NFA)

Neutrophil superoxide production was assessed by *in vitro* neutrophil functional assay upon RF exposure in the order of 850 MHz and 1200 MHz in both the breeds. No significant variation in superoxide production by neutrophil was observed in all the test groups. The results of lymphoproliferation and neutrophil superoxide production were shown side by side to compare the effects in each case (Table – 2).

DISCUSSION

In our study, we assessed the effect of short-term radiofrequency exposure, *in vitro*, on lymphocytes of two rural poultry breeds by detecting mRNA expression of hsp70 gene. Moreover, functional activities (i.e. superoxide production and proliferating ability) of immune-effector cells, viz neutrophil and lymphocyte were assessed through neutrophil functional assay (NFA) and lymphoproliferation assay (LPA), respectively.

The expression of hsp70 mRNA in radiofrequency exposed groups was due to the effect of non thermal, non ionizing radiation fields as no expression of such gene was observed in unexposed groups which was incubated at 37°C. The expression of hsp70 gene was well the stress gene hsp70 upon *in vitro* culture of lymphocytes of both the breeds as compared to higher RF documented in human glioblastoma derived cell-lines (U87MG) in response to exposure of radiofrequency field (Chauhan et al., 2007). In the present study, lower radiofrequency exposure (850 MHz) caused early expression of exposure (1200 MHz) indicating lower RF exposure was more stressful to immune effector cells especially on peripheral lymphocytes. It was also supported by LPA. As, the production of heat shock proteins is triggered electromagnetically it needs 100 million fields remove calcium ions bound to the membranes of living cells, making them more likely to tear, develop temporary pores and leak, as the calcium ions have time to be pulled clear times less energy than when triggered by heat (Blank and Goodman, 2000). Further, weak electromagnetic of the membrane and replaced by potassium ions before the field reverses and drives them back (Diem et al., 2005). The potassium ions resonate, absorb the field's energy and convert it to energy of motion which increases their ability to replace calcium on cell membranes (Liboff et al., 1990). Here, pulses and square waves work best because they give very rapid changes in voltage that catapult the calcium ions well away from the membrane and then allow more time for potassium to fill the vacated sites, where sine waves are smoother, spend less time at maximum voltage, and so allow less time for ion exchange (Andrew, 2007).

Time difference of expression of hsp70 gene in two breeds was also observed in two different RF exposures. It might be due to polymorphism of nucleotide sequences of hsp70 genes which was earlier demonstrated by Mazzi et al. (2003) of different poultry breeds having different heat tolerance capacity.

Duration (Minute)	RIR bird				HB bird			
	850 MHz		1200 MHz		850 MHz		1200 MHz	
	LP	NSP	LP	NSP	LP	NSP	LP	NSP
Control (unexposed)	1.19ab ± 0.08	0.16 ± 0.03	1.19ab ± 0.08	0.16 ± 0.03	1.17ab ± 0.08	0.16 ± 0.04	1.17ab ± 0.08	0.16 ± 0.03
5	0.75c ± 0.15	0.17 ± 0.04	0.85bc ± 0.16	0.17 ± 0.05	0.75d ± 0.14	0.19 ± 0.05	0.79cd ± 0.15	0.18 ± 0.06
15	0.92bc ± 0.14	0.16 ± 0.03	0.77c ± 0.18	0.15 ± 0.03	0.82cd ± 0.13	0.19 ± 0.06	0.91c ± 0.13	0.17 ± 0.05
25	1.17ab ± 0.17	0.15 ± 0.04	0.91abc ± 0.15	0.16 ± 0.02	1.08ab ± 0.10	0.19 ± 0.05	1.07bc ± 0.21	0.18 ± 0.02
40	1.38a ± 0.09	0.16 ± 0.02	1.19ab ± 0.09	0.18 ± 0.06	1.04bc ± 0.06	0.19 ± 0.04	1.11abc ± 0.07	0.18 ± 0.06
60	1.12ab ± 0.11	0.17 ± 0.05	1.26a ± 0.08	0.16 ± 0.05	1.33a ± 0.04	0.19 ± 0.05	1.23a ± 0.07	0.18 ± 0.04
P Value	0.02*	1.00	0.02*	0.98	0.00**	0.99	0.02*	1.00

Table 2: Effect of Radio Frequency on Lymphocytic Proliferation (LP)# and Neutrophil Superoxide Production (NSP)##

Values are expressed as stimulation index ± SEM; ## Values are expressed as Optical density (O.D.) ± SEM
 RIR - Rhode Island Red breed poultry bird, HB - Haringhata Black native poultry bird.; MHz - Mega Hertz; ** $P \leq 0.01$ * $P \leq 0.05$ Means having different alphabet in the same column differ significantly

A typical trend of initial significant ($P \leq 0.05$) decrease (5 minutes through 15 minutes post exposure) followed by significant ($P \leq 0.05$) increase in lymphoproliferation was observed from 25 minutes to 60 minutes *in vitro* post exposure. This trend was observed in both 850 MHz and 1200 MHz RF exposure and in both the breeds. It appears that, short duration RF exposure (at least up to 15 min) rendered considerable stress to the lymphocytes leading to inhibition in proliferation.

However, initial stress could have been coped up during long depuration RF exposure (for 25 min and more) where SI of test samples showed nearly similar values to the control. This finding corroborated with the earlier opinion of Simkó and Mattsson (2004) in respect of direct activation of mononuclear cells (PBMC) by short-term exposure of electromagnetic field (EMF) that leads to proliferation and consequently, hsp70 expression. They reviewed the relevant literatures extensively

and noted that EMF exposure is able to perform such activation by means of increasing the levels of free radicals.

Earlier several workers measured *in vitro* superoxide (reactive oxygen species, ROS) production by neutrophil upon RF exposure. Akdag *et al.* (2007) and Simkó (2007) demonstrated that increased electromagnetic field (EMF) exposure can modify the cellular balance by generating ROS. Physical processes at atomic level were indeed the basis of reactions between biomolecules and EMFs, as the field can magnetically affect chemical bonds between adjacent atoms and alter the energy levels and spin orientation of electrons. Overproduction of ROS can damage cellular components, mainly lipids in membranes and nucleic acids. Moreover, ROS can harm cells by depleting enzymatic and/or nonenzymatic antioxidants triggering progressive dysfunction and eventually genotoxic events (Kong and Lin, 2010). This redox-related mechanism had been mainly documented for the ELF-EMFs (Extremely Low Frequency fields, between 1 Hz up to 100 kHz – electromagnetic fields). Scaiano and co-workers (1994) first proposed that ELF-EMFs exposure can stabilize free radicals in such a way as to increase their lifetime and permit a wider dispersion rather than their return to the basal level. However, in the present study, no significant variation was observed in superoxide production of neutrophil in all test groups. This might be due to the RF exposure used in the study in the order of 850 MHz and 1200 MHz, much higher rate (8000 times) than the above cited observations, which might be unable to affect the chemical bonding of the biomolecules by magnetic spinning. In this context, it may be argued that for better understanding of biological effects of EMF-r on living cells, investigation should be carried out in broader perspective considering *in vivo* situations, especially in relation to human health. *In-vitro* studies encompassing energy absorption due to mobile phones i.e. RF fields below the recommended limits (2 W/kg) did not identify reproducible effects by which cellular dysfunction in living systems could be explained. However, several studies have linked exposure to extremely low frequency electromagnetic fields (ELF-EMF) and increase the risk of childhood leukemia (Ahlbom *et al.*, 2000; Foliart *et al.*, 2001 and Foliart *et al.*, 2002). International Agency for Research on Cancer (IARC), part of WHO, designated RF-EMF from cell phones as a “possible human carcinogen” Class 2B (WHO, 2011). The exposure to continuous RF-EMF radiation poses a greater risk to children, particularly due to their thinner skulls and rapid rate of growth. Also at risk are the elderly, the frail and pregnant women (Cherry, 2001). DNA damage via free radical formation inside cells has also been recorded (Lai and Singh, 1996). Free radicals kill cells by damaging macromolecules such as DNA, protein, and membrane are carcinogenic. In fact, EMR enhances free radical activity. This invisible health hazard pollution (IHHP) is a relatively new environmental threat.

In conclusion, lower radiofrequency emitted by mobile phone had more stressful effect on *in vitro* exposed cells in both the breeds of poultry as indicated by expression of hsp70 gene and initial suppression in proliferation ability of immune-effector cells.

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