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ORIGINAL ARTICLE

Decrease in extracellular collagen crosslinking after NMR magnetic field application in skin fibroblasts

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Abstract Although biological effects of electromagnetic fields were investigated intensively, there is still no agreement on the significance of their effects. The underlying mechanisms and therapeutic importance are still mostly unknown too. In this study, primary cultures of human dermal fibroblasts were exposed to magnetic field at nuclear magnetic resonance (NMR) conditions for in total 5 days and 4 h/day. Among the investigated parameters were: cell proliferation rate, cell morphology, total protein concentration as well as content of skin-specific collagen types I, III, IV. NMR exposure induced distinct changes both in cellular and extracellular components. The extracellular matrix (ECM) of NMR-exposed cells had less cross-linked collagen. In particular, the increase of collagen of the soluble fraction was at 17.2 ± 2.9% for type I, 27.0 ± 1.86% for type III, 17.3 ± 1.46% for type IV ($N = 6$). In the absence of resonance frequency, the effects of magnetic field on ECM were less profound.

Keywords Nuclear magnetic resonance · Skin fibroblasts · Collagen cross-linking

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1 Introduction

The extracellular matrix (ECM) is the largest component of normal skin and gives the skin its unique properties of elasticity, tensile strength and compressibility. On a weight basis, the tensile (breaking) strength of normal skin approaches that of steel, yet skin also has substantial elasticity and compressibility. The ECM is composed of a variety of polysaccharides, water and structural proteins [28]. In addition to serving as a scaffold or structural support for cells, the ECM regulates cellular functions via cell adhesion, lubricates cells and provides a transport system for nutrients and waste products [2]. It regulates also the cell's environment and nutrition through internal circulation of water and is part of the immune system [26]. Misbalances in ECM and collagen metabolism caused by solar irradiation, infections, nutritional disorders and aging processes are of big medical and cosmetological importance [10, 17].

Collagen in the dermal matrix is composed primarily of type I (80–85%) and type III (8–11%) collagens, both of which are fibrillar or rod-shaped collagens [20]. The tensile strength of skin is due predominantly to these fibrillar collagen molecules. The collagen molecule that is secreted by the fibroblast, after hydroxylation, is referred to as procollagen. After the amino- and carboxy-terminal peptides are removed, the molecule is termed collagen [20]. Skin aging has been shown to increase the covalent cross-linking of collagen which is at least partially reversible. This has profound effects on the physiochemical and biological properties of structures [10, 29, 36, 37].

Extracellular matrix degradation, synthesis and repair are subject to fine regulation both by chemical

agents (such as cytokines and growth factors) and physical agents, principally mechanical and electromagnetic stimuli [3]. Since the turn of the twentieth century, a number of electrotherapeutic, magnetotherapeutic and electromagnetic medical devices have emerged for treating a broad spectrum of trauma, tumours and ECM-affecting infections with electromagnetic fields [22, 23, 25]. Many thousands of papers have in fact been published on biological effects of electromagnetic fields, much of it focused on the effects of radio-frequency and microwave fields [1, 7, 11, 13, 21, 27, 38]. In spite of many papers reporting positive effects of pulsed magnetic fields (PEMF) on processes like wound healing [9, 14, 16], pain-killing [12, 34, 35], tissue microcirculation [4, 33], bone metabolism regulation [8, 15, 31, 32], the underlying mechanisms are still mainly unknown. This is partly because electrochemical processes of the human body are extremely complex. They are incompletely understood, and physical effects of electromagnetic fields cannot be ruled out. This indicates the importance of further studies investigating the therapeutic potential of electromagnetic fields. Since skin forms the outermost cellular layer of bodies, it is exposed to electromagnetic fields to an higher extend than other parts of our body that are generally better shielded by the conductive fluid layer beneath our skin.

Our study was designed to evaluate physiological effect of electromagnetic field at nuclear magnetic resonance conditions (NMR) on human skin fibroblasts in culture. The aim of the study was to evaluate NMR exposure effects on ECM component changes (mainly collagen), the level of hydration and the cross-linking level of ECM macromolecules. Furthermore, to investigate the possible NMR influence on proteomic profiles of in-vitro cultured fibroblasts.

2 Materials and methods

2.1 Fibroblast culture

Primary cultures of normal human dermal fibroblasts (NHDF) from PromoCell Co. (Cat. No. C-12302) were used for the experiments. According to supplier's information, the cells were obtained from healthy 60 to 75-year-old individuals. Cells were stored in liquid nitrogen –80°C. Thawing cells was performed by putting them directly in a 37°C water bath. The cells were cultured in specialized fibroblasts growth medium 2 (order No. C-29910) in 25 cm^2 fibronectin-precoated plastic culture flasks until the necessary amount of biomass was accumulated. After centrifugation (5 min, 1,000 rpm) and medium removal by aspiration, the cells were pipetted with fresh medium. Ten millilitre of the suspension was added to a tissue culture flasks and placed into a CO_2 incubator at 37°C for approximately 1 day. Prior to experiments, the cells were pooled together in culture medium and re-distributed uniformly into 18 flasks. Cell morphology and monolayer confluency were checked every 12 h.

2.2 Experimental groups

The three experimental groups were control, cells exposed to constant magnetic field and cells exposed to NMR field. Each group consisted of six culture flasks analysed independently.

2.3 NMR magnetic field application

A quasi-static magnetic field with strength 4 mT was applied (earth magnetic field strength is at 0.7 mT). Nuclear magnetic resonance was generated with a 1 mT and about 100 kHz field. Because of static magnetic field inhomogeneities, a 40 Hz sinusoidal magnetic field was modulated on top enabling NMR in all over the culture chamber. NMR magnetic fields were applied to the samples for in total 20 h during 5 days for 2 h in the morning and 2 h in the afternoon at room temperature. Simultaneously, the control (untreated) group was placed under the safety hood keeping temperature and humidity conditions the same for all groups.

2.4 Sample fractionalization for analysis

For each exposure group, the samples of culture media, cell biomass and extracellular matrix were investigated separately. The cells were detached from the surface by short (1–2 min) treatment with standard trypsin solution. Trypsin in known to cleave peptides on the C-terminal side of lysine as well as arginine residues and therefore does not degrade collagen. Suspended cells were collected and concentrated by gentle centrifugation (800*g*).

The extracellular matrix collagen was fractionalized based on its solubility in 1 M NaCl and 0.5 M acetic acid. Thus, five protein-containing fractions (5 ml each) were obtained for each experimental flask:

1. Culture medium samples were collected after each cell passage.
2. NaCl soluble-ECM fraction: Soluble (not cross-linked) collagen fraction in ECM was collected by

overnight extraction by Tris–HCl buffer pH 7.8 containing 1 M NaCl.
3. Cell biomass: Proteins from cell biomass were extracted by CHAPS–Urea extraction buffer (8 M Urea, 2% CHAPS, 50 mM dithiothreitol (DTT), 0.2(w/v) BioLyte® ampho-lytes, bromophenol blue (traces).
4. Acetic acid-soluble ECM fraction: Semi-soluble (partially cross-linked) collagen fraction was collected by overnight extraction by 0.5 M acetic acid solution (pH 2.5, room temperature).
5. Hot acetic acid-soluble ECM fraction: Insoluble (completely cross-linked) collagen fraction was collected by overnight extraction by 0.5 M acetic acid solution (pH 2.5, 60°C).

2.5 Sample analysis

2.5.1 Total protein determination

The protein content of each sample included control group was determined by Lowry and Bradford methods. A standard curve was prepared by using bovine serum albumin (BSA).

2.5.2 Collagen determination

Sircol™ Collagen Assay (Biocolor Co., UK) was used for collagen quantification according to manufacturer's recommendations. 50 µl aliquots of the samples were mixed in 1.5 ml Eppendorf tubes with 1.0 ml Sircol™ dye reagent containing Sirius Red dye (7,7′-(carbonyldiimino)bis[4-hydroxy-3-[[2-sulfo-4-[(4-sulfophenyl)azo] phenyl]-azo-hexasodium salt) having specific affinity for collagen. The tube contents were gently mixed at room temperature for 30 min and centrifuged at 10,000*g* for 10 min. The collagen-dye pellet at the bottom of the tubes was separated from supernatant, diluted in 1.0 ml of the Alkali reagent and determined photometrically at 540 nm using Bio-Rad Model 680 microplate reader.

2.5.3 ELISA

Specific recognition and determination of different collagen types was performed in indirect ELISA using mouse type-specific anti-collagen antibodies (Calbiochem Co., La Jolla; CA, USA) as primary antibodies. As secondary antibodies, 1:1,000 peroxidase-labelled anti-mouse H&L chain-specific goat IgG were applied (Calbiochem Co.). The collagen binding was visualised by adding 3,3′,5,5′-tetramethylbenzidine (Sigma–Aldrich Chemie Gmbh, Munich, Germany) and measured photometrically at 650 nm.

2.5.4 Electrophoresis (isoelectric focusing and SDS PAGE)

Proteins were extracted by CHAPS–Urea extraction buffer (8 M Urea, 2% CHAPS, 50 mM dithiothreitol (DTT), 0.2(w/v) BioLyte® ampholytes), containing traces of bromophenol blue. Proteins were separated in first dimension by isoelectrofocusing using IEF PROTEAN® system equipped with 7″ IPG-Strips pH 4–7 (Bio-Rad Co., USA) followed by SDS-PAGE in 12% (w/v) gel (Blinnikova et al. 1990). Protein fixation in the gel was done using 12% trichloracetic acid solution (Sigma–Aldrich Chemie Gmbh). The obtained gels were stained with a PageBlue™ solution (Fermentas Co., USA), scanned and corresponding densitometry profiles acquired using image analysis. Respective molecular weights of the bands were estimated by using the DIAdem® software version 8.0 (National Instruments, Austin, USA) using molecular weight markers (SIGMA Co., Fermentas Co., USA) as standards.

For conventional SDS PAGE, proteins were extracted by SDS-DTT extraction buffer (1D SDS PAGE: Tris–HCl pH 7, 0.07 M SDS, 5 mM EDTA, 50 mM DTT, 1 mM phenylmethylsulphonylfluoride), then applied to conventional SDS PAGE 12% and processed like described above.

2.5.5 Statistical analysis

Common statistical procedures were applied to the data using Microsoft® Excel 2003 Software as well as SPSS Software (SPSS GmbH, Munich, Germany). Unless stated otherwise, error bars in figures indicate ± standard deviation. For image analysis, graphic processing algorithms were applied using Visual Builder Module and additional custom made programming in LabView® Software (National Instruments, USA).

3 Results

Averaged protein content profiles in culture media obtained for all used experimental groups are shown in the Fig. 1. The measurement of total protein content provides general information about the intensity of proliferation and metabolic processes in the cultured cells. It was also important to detect and take into account possible deviations in experimental flasks

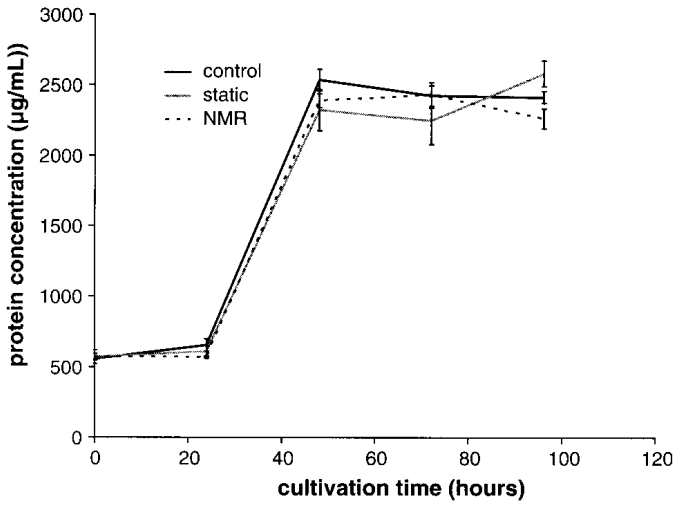

Fig. 1 Total protein content in culture media determined by Lowry method for all experimental groups ($N = 6$) before and after NMR and static magnetic field application. Protein concentration was determined after each culture medium change

because of potential occasional events (infection, contamination etc.). The analysis of the curves in Fig. 1 as well as further protein determinations in different ECM fractions and biomass showed neither NMR nor static field effects on general metabolic activity and/or proliferation rate of dermal human fibroblasts. Nevertheless, further analysis of cellular and ECM fractions with SDS PAGE has revealed distinct effects of NMR on protein composition of the samples. The redistribution of proteins in the soluble ECM fraction after NMR application is shown in Fig. 2. NMR-induced changes were mostly seen in the 40 and 60–85 kDa regions (Fig. 2a) which are typical for collagens. These effects were observed as width as well as density variations in protein bands. They were clearly visible in densitometric profiles (Fig. 2b, marked by arrows). Thus, for soluble ECM fractions, it can be presumed that NMR treatment resulted in a significant decrease of high-molecular weight collagen bands accompanied by an unspecific increase of protein content at low-molecular weight regions. Similar molecular weight-redistribution trends, although shaded by smear, were observed in the electrophoretic profiles of semi-soluble and insoluble ECM fractions.

Comparative studies of collagen content in NaCl soluble (less cross-linked) and acetic-acid soluble (more cross-linked) ECM fractions in type-specific ELISA revealed significant magnetic field effects on all studied collagen types (Fig. 3). In case of NMR field applied, the ECM related effects were much profound as compared to quasi-static field variants. Specifically, for soluble ECM collagens, a noticeable increase in collagen content was detected after NMR application. In particular, increase of collagen in NaCl-soluble fraction has made up 17.2 ± 2.9% for type I, 27 ± 1.86% for type III, 17.3 ± 1.46% for type IV ($N = 6$). The results also showed a significant ($P < 0.05$) decrease in semi-soluble (cold acetic acid-soluble) ECM collagens for collagen type I (15.7 ± 2.5%), type III (19.6 ± 2.1%) and type IV (34.7 ± 2.86%) and significant decrease in insoluble ECM collagens for collagen type III (26.0 ± 2.3%) and type IV (35.0 ± 1.9%) in NMR-exposed samples as compared to control.

In contrast, the cellular collagen checked with ELISA showed that NMR application, independently of the collagen type, did not result in any specific change.

Quite characteristic changes in 2D protein patterns were detected. They indicate specific alterations in the cellular proteome after NMR application as compared to control (Fig. 4). Even rough two-dimensional protein separation allowed revealing multiple appearances/disappearances of protein spots as a result of

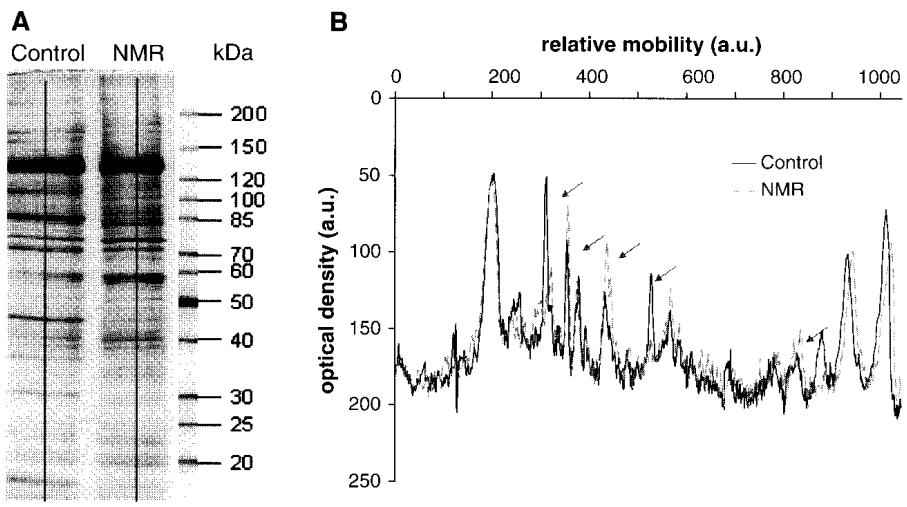

Fig. 2 SDS PAGE analysis of the soluble ECM fraction from control and NMR groups for comparison. Correspondent densitometry profiles are shown on the *left*. The differences between the two protein band profiles are obvious and involves multiple molecular weight regions

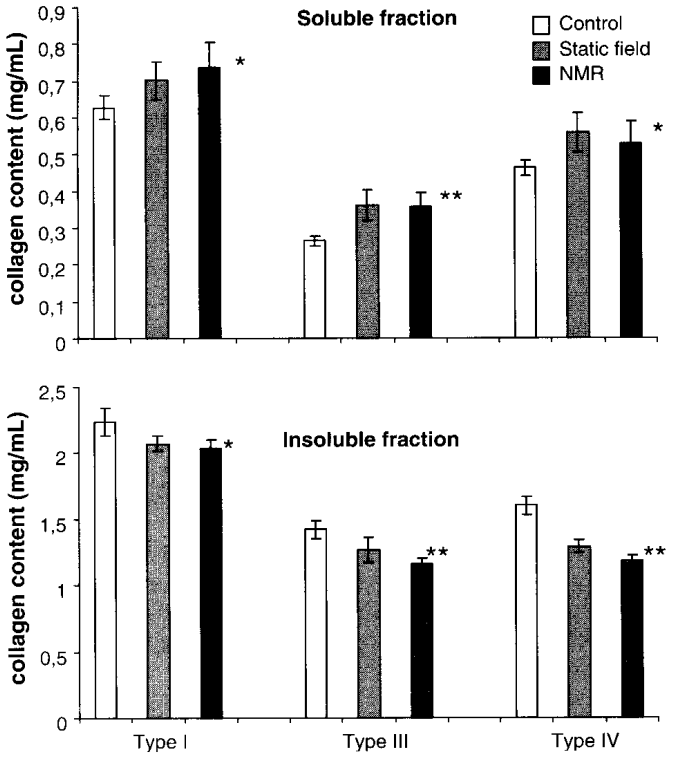

Fig. 3 Relative content of specific collagen types (I, III and IV) for soluble (*above*) and insoluble (*below*) ECM fractions before, with static magnetic field and with NMR applications. In both graphs, the first three bars represent collagen type I, the following triplets—types III and IV, respectively. *Differences to control at $P < 0.05$, **difference to control at $P < 0.01$

NMR application. Since the intensity of spots corresponds to the level of protein expression, it was interesting to match the spots with particular cellular functional activities affected by NMR. Using fibroblasts proteome reference maps, the most affected spots were identified as nexins, profilins, actins, destrins and procollagens, thus belonging to the cytoskeletal/cell adhesion regulation protein group.

4 Discussion

Fibroblasts being mesenchymal cells within the dermatological meshwork are involved in numerous mechanisms, including fibrogenesis, tissue contraction, and tissue skeleton building. They also play a role in epithelial differentiation, leading to collagen formation. After tissue damage has occurred, whether it be through acute forces such as trauma, or chronic forces such as aging, stress, and oxidative damage, the fibroblasts possess an unique ability to change their phenotype thereby contributing actively to the repair process [6]. Successful stimulation of fibroblast to produce more collagen and/or to change the collagen quality is a critical point in rejuvenation of aging skin. As a result of appropriate stimulation, ECM–water balance of the whole tissue may change so that wrinkles can disappear and skin thickness may increase.

There is currently much research going on in this area of skin care. Synthesis and degradation of the most common extracellular protein in mammalian tissues, type I collagen, is under strict physiological control but is affected in several pathological conditions, e.g. osteoporosis, fibrosis, rheumatoid arthritis and various cancers [6, 17, 18]. After activation of the appropriate gene section in the cell nucleus and transcription of the information to mRNA, pro-a-chains are created in the ribosomes and then converted enzymatically to procollagen in the cytoplasm by prolylhydroxylase. Procollagen is ejected from the fibroblasts and is enzymatically modified outside the cell into tropocollagen fibres. These finally juxtapose to insoluble collagen fibres in the extracellular matrix. This complex process can be influenced by different agents at various levels. So far, two approaches have been clinically proven to be successful: (a) stimulating the growth hormone secretion and (b) application of

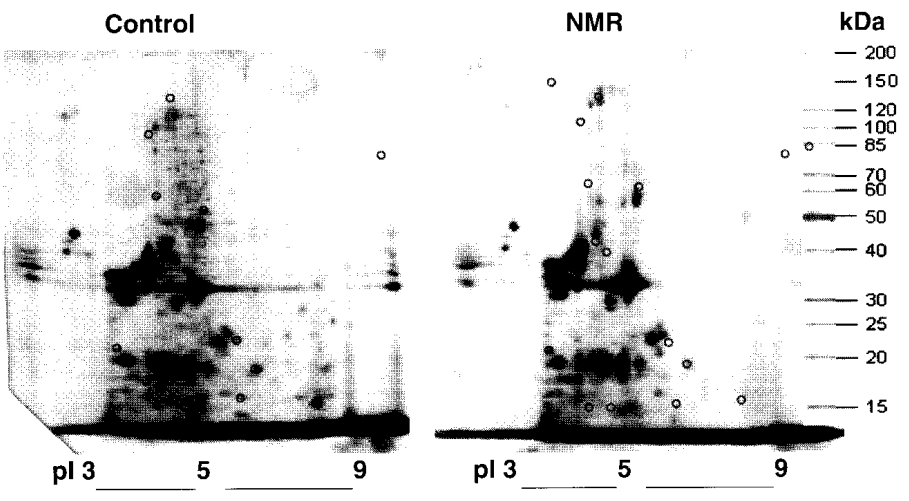

Fig. 4 Comparison of protein expression maps for control and NMR treated samples. The most obvious differences in protein expression between the two samples are indicated by *red circles*. Proteins were separated horizontally according to their isoelectric points (pI) between pI 3 and 10 and vertically according to their molecular weight

electrical or mechanical impulses [24]. Growth hormone promotes fibroblasts to produce more collagen throughout the body and has some obvious disadvantages such as possible pharmacological and immune side effects, high costs, etc.

In many tissues, mechanical and electrical signals may regulate the synthesis of cellular and ECM components by stimulating signalling pathways at the cell membrane [27, 32, 38]. In soft tissue, alternating current electrical fields induce a redistribution of integral cell membrane proteins which, hypothetically, could initiate signal transduction cascades and cause reorganization of cytoskeletal structures [5, 7, 21, 32]. Rodemann et al. [30] reported that long-term magnetic field exposure of fibroblasts in vitro induces the differentiation of mitotic to postmitotic cells that are characterized by differentiation-specific proteins and differentiation-dependent enhanced metabolic activities.

In our study, it was found that NMR exposure induces in fibroblasts distinct changes both in cellular and ECM components. Although, this treatment did not lead to a noticeable increase in the proliferation rate, changes in the metabolism level, in general protein content and in the cellular micro-morphology.

The observed effects of NMR can be marshalled in two groups, (1) protein expression effects and (2) ECM-related effects. The treatment resulted in electrophoretic protein profile changes caused by alterations in protein synthesis. Multiple differences in protein expression after NMR exposure were found by 2D SDS. NMR application resulted in expression and up-regulation of some protein classes and down-regulation of others. After calculations of observed molecular weight and observed isoelectric point, these proteins were preliminary identified as structural and regulatory agents mainly involved in cell adhesion and movement. This can be regarded as an additional testimony for the reported influence of pulsed magnetic fields on protein synthesis [16, 21]. In contrast, a number of investigators have been unable to show any effect of electromagnetic fields on tissue metabolism [4, 14].

Previous in vitro studies have suggested that PEMFs may have the effect of modifying the extracellular matrix by promoting the synthesis of matrix molecules. The synthesis of cartilage molecules is enhanced by PEMF, and subsequent endochondral calcification is stimulated [1]. It was also reported that pulsed electromagnetic fields preserve proteoglycan composition of the extracellular matrix [19].

Pulsating electromagnetic fields have been evaluated in the treatment of soft tissue injuries, with the results of some studies providing evidence that this form of therapy may be of value in promoting healing of chronic wounds (such as bedsores), in neuronal regeneration, and in many other soft tissue injuries. Results of one previous study in an experimental achilles tendinitis model in rats indicated that there were changes in water content in injured tendons treated with pulsating electromagnetic fields [7, 22].

In our experiments, as a response of the cell construct on both static field and NMR exposure, collagen in ECM has turned more soluble (less cross-linked). This induced re-distribution of collagen between soluble and insoluble fractions was observed for all collagen types. Most of the reported effects were less evident in case of quasi-static magnetic field application on the same cell model. Less cross-linked collagen has a higher ability to bind GAGs and water that can be generally considered as a favourable effect counteracting aging-related dehydration. Nevertheless, the particular physiological impact of NMR application on fibroblasts proteome is still to be studied on the base of the analysis of the involved signalling pathways and extensive expression profiling.

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Correction:
2.3 NMR magnetic field application

A quasi-static magnetic field with strength 3 mT was applied (earth magnetic field strength is at 0.05 mT). Nuclear magnetic resonance was generated with a MBST 01 – 300 device, with 3 mT and about 127.45 kHz field. Because of static magnetic field inhomogeneities, a 40 Hz sinusoidal magnetic field was modulated on top enabling NMR in all over the culture chamber. NMR magnetic fields were applied to the sampies for in total 20 h during 5 days for 2 h in the morning and 2 h in the afternoon at room temperature. Simultaneously, the control (untreated) group was placed under the safety hood keeping temperature and humidity conditions the same for all groups.