

## Research Article

# Response of Functional Neutrophils after Exposure to a Novel Immune Chip (Energy Cell)

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Received: February 11, 2019; Accepted: March 23, 2020; Published: March 26, 2020

**Abstract**

Neutrophils are the most abundant type of granulocytes in most mammals. They possess a dual role as phagocytes and as proinflammatory cells. By being present in the bloodstream, they form the first line of cellular defense against invading microbial pathogens as an essential part of the innate immune system. The experiments were conducted with cultivated human promyelocytes which were directly exposed to a novel immune chip during their 6-day differentiation period to functional neutrophils. The same cells, but without the immune chip (= Energy Cell), served as corresponding controls. The samples were cultivated in the same incubator, but separated from each other by several layers of aluminum foil. After differentiation, the growth characteristics, and the oxidative burst of the functional neutrophils without and with exposure to the Energy Cell were examined. Altogether, 5 independent experiments on different days were conducted. In comparison to the untreated controls, the Energy Cell caused an increased cell number by  $17.2 \pm 3.8\%$  and an increased cell diameter by  $9.5 \pm 0.2\%$  (mean values  $\pm$  standard deviations;  $p \leq 0.05$ ). Moreover, the basal cell metabolism of the functional neutrophils as well as the generation of superoxide anion radicals was also increased by  $23.0 \pm 5.5\%$  and  $21.3 \pm 6.2\%$ , respectively (mean values  $\pm$  standard deviations;  $p \leq 0.05$ ). The results suggest that use of the Energy Cell might be able to cause an improved defense against microbial pathogens.

Keywords: immune chip, vital field technology, oxidative stress, functional neutrophils, cell culture

**Introduction**

Neutrophils are the most abundant type of granulocytes in most mammals. They possess a dual role as phagocytes and as proinflammatory cells [1]. By being present in the bloodstream, they form the first line of cellular defense against invading microbial pathogens as an essential part of the innate immune system. Moreover, neutrophils are the first responders of inflammatory cells which migrate towards the site of inflammation [2-6].

Similar to macrophages, neutrophils attack microbial pathogens by activating a so-called respiratory or oxidative burst which is independent from energy-producing metabolic processes and involves the catalytic conversion of dimolecular oxygen into superoxide anion radicals by the NADPH oxidase complex [7]. Secondly, also other strong oxidizing agents with potent antimicrobial properties are generated. However, reactive oxygen species have been increasingly recognized as important components of intracellular signaling [8].

Prompted by this background we investigated the effect of a novel immune chip (= Energy Cell) on the growth characteristics and the oxidative burst of cultivated human promyelocytes which have been differentiated to functional neutrophils in corresponding cultures without and with exposure to the Energy Cell.

**Materials and Methods****Description of the Energy Cell**

Basically, the Energy Cell is influenced by frequency spectra that are generated by special frequency generators. With the so-called vital field technology, electromagnetic fields (up to 120 GHz), microcurrent frequencies (up to 1 GHz) and different magnetic fields are generated via special antennas. The Energy Cell is placed on these antennas and exposed to the specific frequencies for a definite time period. Due to its storage properties, the chip is able to record this information and release it over several months. In the case of the Energy Cell, the stored frequencies of specific immune cells were used and the chip was exposed to this pattern in order to achieve the desired result.

This patented information store is manufactured using a special process after years of development work. A precisely defined proportion of high-quality storage media is poured into a particularly skin-friendly silicone base. This makes it possible to conserve the effect of these frequency spectra as long as possible. Experience to actual data show that the chip remains fully effective for at least 6 months and should be replaced afterwards.

### Cell culture and differentiation of cells to functional neutrophils

Human promyelocytes (cell line HL-60, ACC-3, ECACC 98070106; Leibniz Institute, German Collection for Microorganisms and Cell Cultures, Braunschweig, Germany) were routinely grown as non-adherent cell cultures in 75 cm<sup>2</sup> flasks containing 25 ml of RPMI 1640 with 10% growth mixture and 0.5% of gentamycin and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Subcultures were conducted twice a week for further growth or differentiation. During the experimental period, the cells of every second subculture were differentiated to functional neutrophils by the addition of 1.5% dimethylsulfoxide to the culture medium for 6 days. Then, cells were capable of undergoing an oxidative or respiratory burst upon phorbol ester stimulation (more details, see below). All cell culture reagents were obtained from PAN-Biotech, Aidenbach, Germany.

### Exposure to the Energy Cell

During the cell differentiation period of 6 days, cells were cultivated in the incubator without (= corresponding control) and with the Energy Cell in direct contact to the cell culture flask. The samples in each experiment were separated by several layers of aluminium foil wrapped around the flasks. In addition, the flasks had a distance of about 50 cm to each other in the incubator. 5 independent experiments with cells of different in vitro age at passages 10 to 16 were conducted within a total experimental period of 4 weeks.

### Examination of cell number and cell size distribution

100 µl-aliquots of the suspensions with differentiated cells were diluted directly with 9,900 µl of CASYton, a specific isotonic measurement buffer for the CASY cell analyzer system (OLS - Omni Life Science, Bremen, Germany). The device was equipped with a 150 µm capillary tube and only counts in the range between 7.5 and 30 µm were taken for evaluation by setting of the cursors. The basic principle of the CASY device is the use of the electronic current exclusion (ECE) technology. Particles are aspirated by the capillary which generates an electronic signal. The intensity of the signal correlates directly with the particle size and conductivity. As a result, CASY detects precisely the volume of cells, independent of their shapes [9-11]. Moreover, viable and dead cells (= debris) can be detected by their different volumes. Viable cells, with a polarized membrane generate a high resistance signal which correlates with the volume of the cell. Dead cells have a much lower resistance due to their disrupted cell membrane.

### Preparation of cells after differentiation

For the experiments, functional neutrophils were collected by centrifugation (5 min at 190 x g), washed twice with phosphate-buffered saline by resuspending and centrifugation and were finally resuspended in phosphate-buffered saline with calcium and magnesium containing 10 mM glucose. Cell suspensions were stored at room temperature for not more than 60 min before experiments were executed.

### Basal energy metabolism of functional neutrophils

60 µl of cell suspension were added to a reaction mixture

consisting of 120 µl phosphate-buffered saline with calcium and magnesium consisting of 30 mM glucose and 20 µl of WST-1 as tetrazolium dye (all reagents were from Sigma-Aldrich, Deisenhofen, Germany). The cleavage of the dye is directly related to the activity of the basal cellular metabolism, which also includes the generation of adenosine triphosphate in mitochondria. The optical density was continuously recorded by an Elisareader (BioTek SLx808 with software Gen 5 Version 3.00) as a differential wavelength measurement  $\Delta OD = 450$  minus  $690$  nm and analyzed with Microsoft Excel for the time interval 0 to 40 minutes.

### Generation of superoxide anion radicals by functional neutrophils

60 µl of cell suspension were added to a reaction mixture consisting of 100 µl of phosphate-buffered saline with 30 mM glucose, 20 µl of phorbol 12-myristate 13-acetate solution and 20 µl of WST-1 as tetrazolium dye (all reagents from Sigma-Aldrich, Deisenhofen, Germany). As already described in detail previously [12], the course of superoxide anion radical inactivation as produced by the stimulated functional neutrophils was monitored by cleavage of the dye [13-15]. A differential measurement of the optical density at  $\Delta OD = 450$  minus  $690$  nm for 30 min at 37°C using a BioTek ELx 808 ELISA reader with software Gen 5 Version 3.00 was performed.

### Statistical analysis

Statistical analysis of the cell biological data was done by using the parameter-free two-sided Wilcoxon-Mann-Whitney test and assumed as significant at  $p \leq 0.05$ .

### Results

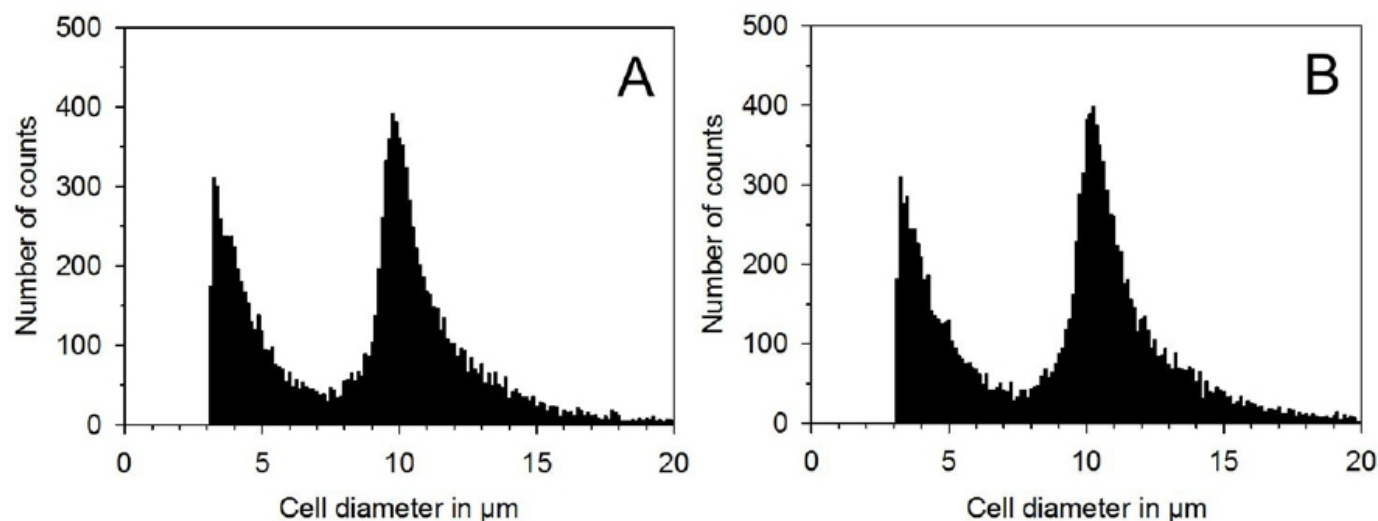
#### Effect of the Energy Cell on cell number and cell size distribution

Since the cell numbers varied from experiment to experiment and ranged from 300,000 to 800,000 cells/ml suspension, we decided not to use the absolute cell numbers for further evaluation, but the relative values in comparison to the corresponding untreated control in each experiment. Taken together for all 4 independent experiments, the cell numbers were increased by the Energy Cell in comparison to untreated controls by  $17.2 \pm 3.8\%$  (mean value  $\pm$  standard deviation). This increase was statistically significant ( $p \leq 0.05$ ).

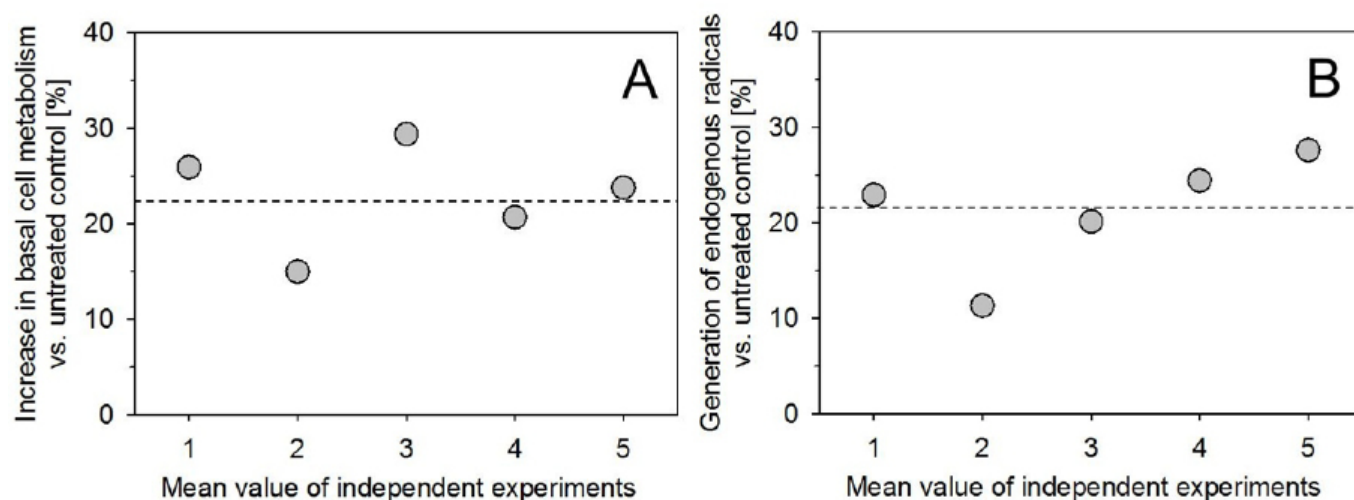
Independent from the cell numbers achieved after the differentiation process, the peak cell diameter was also increased by the Energy Cell in all experiments from  $9.5 \pm 0.2$  µm to  $10.4 \pm 0.3$  µm ((mean value  $\pm$  standard deviation). This is equivalent to a relative increase of  $9.5 \pm 0.2\%$  and is also statistically significant ( $p \leq 0.05$ ). This shift in peak cell diameter can be clearly seen in Figure 1.

#### Effect of the Energy Cell on the basal energy metabolism and generation of superoxide anion radicals by functional neutrophils

As depicted in Figure 2A, the use of the Energy Cell resulted in an increased cell metabolism of functional neutrophils in all 5 independent experiments when compared with the untreated controls. Alltogether, the increase of basal cell metabolism



**Figure 1.** (A) Effect of the Energy Cell on peak cell diameter of human promyelocytes after a 6-day differentiation process to functional neutrophils. (B) For comparison, see the peak volume of unexposed controls. Note the increased peak cell diameter for exposed cells. Data represent the original data of one representative measurement. For further details, see text.



**Figure 2.** (A) Increased basal cell metabolism of functional neutrophils after exposure to the Energy Cell for 6 days during the differentiation process from promyelocytes. Data represent mean values of each independent experiment ( $n = 5$ ). The dashed line gives the mean increase of all experiments. (B) Generation of superoxide anion radicals by functional neutrophils after exposure to the Energy Cell for 6 days during the differentiation process. Data represent mean values of each independent experiment ( $n = 5$ ). The dashed line gives the mean increase of all experiments. In both graphs, the untreated controls are set as 0%.

was  $23.0 \pm 5.5\%$  (mean value  $\pm$  standard deviation), which is statistically significant ( $p \leq 0.05$ ).

In accordance with the increased cell metabolism caused by the exposure to the Energy Cell, was the increased generation of superoxide anion radicals of the exposed cells (Figure 2B), which was in the same range. The mean value  $\pm$  standard deviation of the increase of all 5 independent experiments was  $21.3 \pm 6.2\%$  ( $p \leq 0.05$ ).

## Discussion

The Energy Cell markedly enhanced proliferation of promyelocytes during their differentiation process to functional neutrophils. Therefore, the number of cells able to undergo phagocytosis as the first defense against microbial pathogens has been increased in vitro. However, the increase in peak cell diameter by the Energy Cell is difficult to explain. From our experience, an increased proliferation of cells is related to an increase in cell metabolism and might also increase cell diameter/cell volume.

However, an increase in cell diameter/cell volume can be also observed for cells in the initial stage of apoptosis [16-18]. More investigations are needed to come to a closer understanding why the Energy Cell caused the observed cellular alterations.

Reactive oxygen and nitrogen species ubiquitously exist in complex biological systems, tissues or cells to participate in various cellular signaling pathways [19-22]. Moreover, they play an important homeostatic role in regulating the signal transduction involved in cell proliferation and cell survival.

The examination of oxidative stress due to an excess of reactive oxygen radicals was not a point of interest in this study. We were mainly interested to investigate whether the cells exposed to the Energy Cell during a 6-day differentiation period might react different in comparison to untreated cells. We found that exposed cells generated significantly more superoxide anion radicals than unexposed cells. Speaking in terms of a primary defense against microbial pathogens, this is a very promising result, because the increased generation of the total number of

defending cells as well as the increased generation of superoxide anion radicals might also result in a more efficient and powerful first defense against microbial pathogens [23-25].

### Conclusion

The present study demonstrates that exposure of human promyelocytic cells to the Energy Cell during their differentiation to functional neutrophils resulted in an increased number of cells as well as in an increase of generation of superoxide anion radicals. The results suggest that use of the Energy Cell might be able to cause an improved defense against microbial pathogens.

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To cite this article: Dartsch PC . Response of Functional Neutrophils After Exposure to a Novel Immune Chip (Energy Cell). *Japan Journal of Medicine.* 2020; 3:2.

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