BBEMG

Research methods

Prepared by Belgian BioElectroMagnetics Group (BBEMG)

Preliminary remarks

1 EMF studies and the interpretation of the results: A single study is not enough!

Results of a single study cannot be used to draw conclusions. They can only be used to emit hypothesis, that will need to be confirmed by the replication of this study and by other studies:

Epidemiological studies are theoretically the most valuable research method in terms of public health. In reality this is not so: epidemiology provides correlation but not causal relationships. Other studies are therefore necessary for a better understanding of pathophysiological working mechanisms and to enhance the credibility of epidemiological studies:

- controlled **human clinical trials**,
- ***in vivo* investigations** and
- ***in vitro* studies**.

Well done ***in vitro*** studies may unravel cellular and molecular working mechanisms which can explain pathophysiological effects.

On the contrary, the results of *in vitro* studies do not necessarily mean that observed effects will be the same *in vivo*.

2 Biological effect or health risk?

Biological effects are measurable modifications in response to a stimulus (e.g. exposure to electromagnetic fields or sunlight). Biological effects are not necessarily harmful to health: reading a document produces a biological effect, but it is not a harmful activity to health.

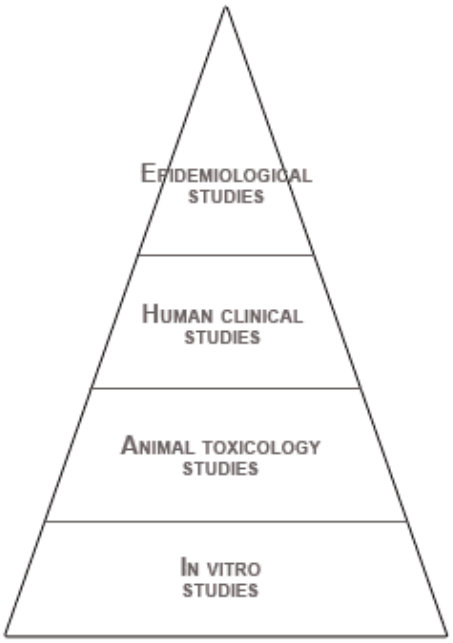
The human body possesses compensation and regulation mechanisms for many stimuli. There is a **health risk** whenever the biological effect exceeds the capacity of normal compensation of the organism, thereby resulting in a deterioration of health.

Note: Confusion between biological effect and electromagnetic compatibility

The correct functioning of an electrical appliance can be disturbed by an electromagnetic field emitted by a nearby electrical device. The disturbances caused by this field are called **electromagnetic interferences**. To avoid such interference, it is necessary to be aware of the **electromagnetic compatibility** of electrical appliances.

It is important not to confuse **biological effects** with **electromagnetic field interferences** from an **electronic device**. Certain materials are very sensitive to low-frequency magnetic fields. For example, a computer screen can be disturbed by a magnetic field of about 1 µT. The interference is due to the refresh rate of the display on the screen. This frequency is indeed close to 50 Hz.

They are several methods to study potential effects of 50 Hz electric and magnetic fields on health:



- Epidemiology - Research for the existence of a statistical association between a given factor and the appearance of a disease
- Human studies - Volunteers are subjected to 50 Hz electric and magnetic fields for short periods of time, under controlled exposure. Multiple functions are analysed
- In vivo studies - Research of effects of electric and magnetic fields on animals
- In vitro studies - Research for mechanisms of action of electric and magnetic fields on cells
- Modelling - Computer simulation of electromagnetic fields, for example to estimate the distribution of ELF fields induced in the human body by overhead power lines or contact currents

Epidemiology

Epidemiology is an observational science. Its purpose is to examine hypotheses dealing with the distribution and causes of disease onset in a given population. An epidemiological study searches for a statistical association between a given factor and the emergence of a disease, and then determines the importance of this association. Epidemiological studies are sensitive to several types of bias.

Advantages of epidemiological studies

- Focus = humans
- Exposure to agent = real situation
- Endpoints: mortality & morbidity
- Potentially hypersensitive subjects can be investigated
- Acute and chronic exposures can be studied

Limitations of epidemiological studies

- Difficult to demonstrate causality
- Difficult to take all ‘confounders’ into account
- Difficult to obtain accurate individual measurements of real exposure
- Epidemiological studies are very expensive and time consuming (especially cohort studies)

1 Principles of epidemiological studies

To study the influence of the factor "electric field" and/or "magnetic field" on a disease, the epidemiologist carries out investigations on relevant populations. Two main types of epidemiological studies are usually carried out: analytical and experimental (trials) epidemiology.

1.1 Analytical epidemiology

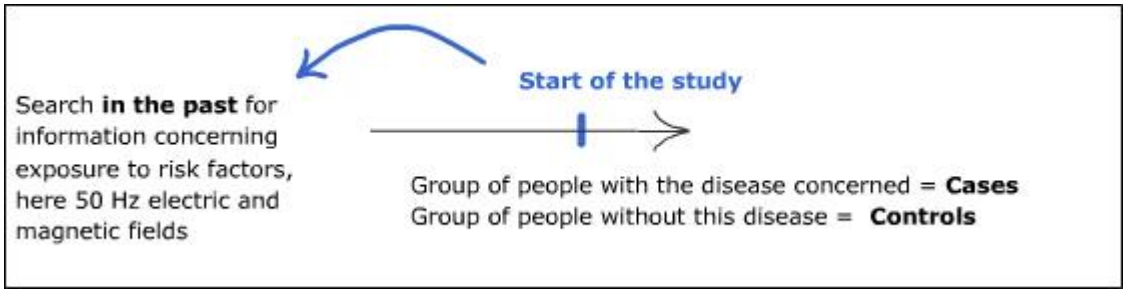
a. Ecological studies

Ecological studies focus on the comparison of groups, rather than individuals: they study the association (correlation) between exposure variables and health, when researchers do not have any individual data.

Ecological studies do not reflect the exposure or the health of each individual in the group but the average level of exposure and the health of populations. Researchers can study the same population at different times (temporal variations) or more populations of different geographical areas during the same period (geographic variation). For example, this kind of survey can be used to study the relationship between the concentrations of air pollutants (CO_2, ozone...) and mortality collected in the following days from hospital data and death certificates.

Even if the results are not precise at individual level, ecological studies are interesting because they are quickly set up and quite inexpensive since based on already existing data. It can provide the base to build other studies as case control or cohort studies. They can generate hypotheses.

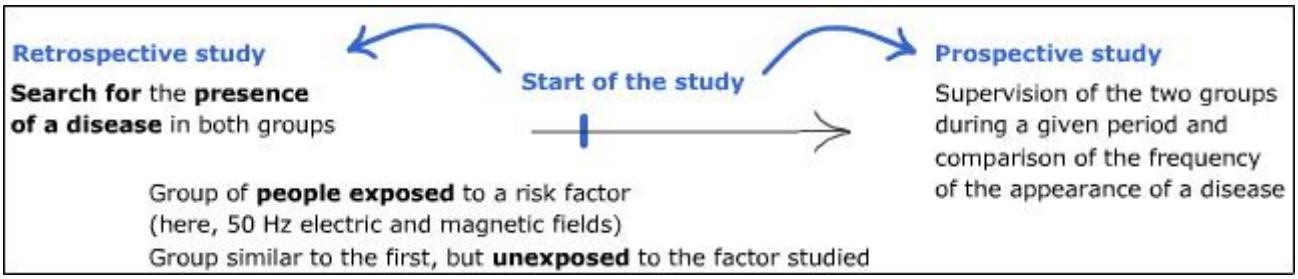
b. Case control studies



One selects a group of subjects that have the studied disease (cases), and a group of subjects without this disease (controls). For each subject of the investigation, one will search for information concerning exposure to the risk factors in their relevant past. For this reason, case control studies are qualified retrospectives, since the studied disease has already occurred when one searches for the earlier exposure to the risk factor. One then compares the exposure to the risk factor in both cases and controls. The advantage of this protocol is that it is inexpensive and feasible within a short time. Its principal disadvantage comes from the difficulty of rebuilding the story of cases and controls in a comparable way and without bias. The measurement of the association is called the odds-ratio (OR). Case-control studies are a good type of study for rare diseases.

c. Cohort studies (exposed versus non exposed)

During a given period, one supervises a group of people exposed to a risk factor as well as a group similar to the first, but not exposed to the studied factor. The appearance frequencies of the disease in the two groups are compared.



- **Retrospective cohort studies:**

  In retrospective studies, researchers are looking in the past for the existence of a disease in two groups of people as alike as possible excepted for their exposure to EMF. In retrospective cohort studies, the measurement of association is called the relative risk (RR).

- **Prospective cohort studies**:

  Because researchers are waiting for the appearance of the disease with the passage of time, this type of study is called a prospective study.

  The advantage of prospective cohort studies is that it allows a better control of bias. Disadvantages are the high cost and difficulty of carrying out this type of study when the disease is rare or occurs after a long latency period. The measurement of association is called the relative risk (RR).

1.2 Trials: Experimental epidemiology

The term “Experimental” means that, contrarily to cohort studies, researchers control the exposure conditions of the subjects. Groups exposed and unexposed are monitored and compared with respect to the impact of the event studied. The assignment of a subject to a group or the other is randomized.

When properly conducted, these studies represent the ideal model to study the relationship between exposure to an agent and the occurrence of a disease, since the groups being compared differ only by one characteristic: the exposure. However, this approach is not always possible, often for ethical reasons if the exposure to which a group of subjects should be submitted is potentially harmful. Trials are mostly used to control the effectiveness of interventions (e.g. drugs).

2 What is a meta-analysis?

Meta-analysis is a statistical technique that **gathers** the data of comparable epidemiological studies in order to analyse them and **evaluate the coherence** of the obtained results.

3 Significant risk

The **odds ratio** (case control studies) corresponds to the risk exposure of cases compared with the risk exposure of controls.

The **relative risk** (exposed - unexposed studies) corresponds to the risk of exposed people to the studied factor compared with the risk of non-exposed people.

If the odds ratio or the relative risk is equal to 1, this indicates a lack of increased risk in the group of cases or the exposed population. The closer the odds ratio or relative risk is to 1, the **lower** the risk.

The confidence interval indicates the degree of accuracy with which one measures the odds ratio or the relative risk. A 95 % confidence interval (CI 95 %) means that this interval contains the true value of the relative risk or odds ratio with a probability of 95 %. The odds ratio or the relative risk is considered as **significant** when **the confidence interval does not contain the value 1**.

Example: If a relative risk is equal to 2.7 with a confidence interval at 95 % of (2.3 - 3.1), the risk is significant, since the lower limit of the interval is higher than 1. On the other hand, a relative risk of 1.4 with a confidence interval to 95 % of (0.9 - 1.9) is not significant because the value 1 is contained in the confidence interval.

4 Association and causality

Epidemiological studies cannot determine a clear relationship of cause and effect. If one finds an association between a factor and a disease, that does not mean that this agent caused the disease. Establishing a relationship of cause and effect requires the checking of several criteria:

- **strength of the association**: the causal nature of an association will be all the more probable since the value of the relative risk or the odds ratio is high;
- **specificity of the association**: a given exposure specifically involves a given pathology;
- **constancy of association and reproducibility**: it is necessary to find the same results in several investigations and different populations;
- **coherence** with results of the studies already published in the scientific literature;
- **temporal relation**: exposure to the alleged causal factor must precede appearance of the disease;
- **dose response relationship**: the more significant the exposure, the greater the probability of an effect on health;
- **plausibility** of the biological mechanism highlighted.

5 Bias of epidemiological studies

5.1 Information bias

Information bias concerns the estimation and measurement of parameters that influence the living organism. After 30 years of research, scientists have not managed to establish factor(s) of exposure to be studied in order to understand biological effects:

- **What should be measured or calculated?** electric field, magnetic field, electrical consumption, wiring code...
- **Which parameters should be measured?** peak, average, median, an accumulated dose...
- **How long is it necessary to measure?** specific measurement, 24h, 1 week...
- **Where is it necessary to measure?** inside the house, in front of the house, in the bedroom, at the workplace …
- **When is it necessary to measure?** during the day, during leisure time, at night
- **Is the continuous or variable character of our exposure significant?**
- **Which threshold should be chosen?** 0.2 µT? 0.3 µT? 0.4 µT or higher?

Many studies are carried out with a threshold of 0.2 µT. Dr David Savitz first chose this threshold of 0.2 µT to establish a distinction between exposed people and non-exposed people (Savitz, 1988). The goal was not to define a level of security but to establish a threshold for the study (Lynch C, 1997). The studies that followed were carried out with this threshold or other thresholds: 0.3 µT, 0.4 µT.

5.2 Selection bias

This relates to:

- the under-representation of subjects of under-priviledged socio-economic levels when the choice of controls is carried out by drawing names from a telephone list,
- for certain studies, there is a need for a given stability of housing for the controls: this involves lesser mobility of the controls than of the cases,
- refusal to answer a questionnaire or to authorize the measurement of fields inside the residence: non respondents can then be different from those who agree to take part in a study.

5.3 Confusion bias

For **domestic exposure**, this bias primarily refers to the studies concerning the evaluation of fields radiating from surrounding power lines. High tension power lines are not laid out randomly in cities: they are often located in places where traffic congestion is considerable, air pollution is significant, and socio-economic status is low. Potential confounding factors (e.g. physical, chemical, genetic, nutritional, etc.) are numerous.

In the **occupational environment**, potential confounding factors frequently occur. In addition to the usual factors, such as socio-demographic characteristics, smoking, alcohol consumption, or general employment conditions, few studies consider factors such as

organic solvents, poly-chlorinated biphenyls, welding fumes, or ionising radiation, which often characterize jobs exposed to electromagnetic fields (Knave B, 1988 and Gallagher RP, 1990).

5.4 Publication bias

Epidemiological studies suggesting an association are generally published in the scientific literature. On the other hand, epidemiological studies indicating a lack of association are not consistently published.

Human studies

In epidemiological studies, it is impossible to isolate the possible effects of magnetic fields from those of electric fields. However, the controlled conditions of the studies undertaken in the laboratory make it possible to analyse the isolated or compound effects from these two types of fields. The results of cellular and animal studies indicate that the most probable site affected by the action of electric and magnetic fields is the central nervous system.

Studies on volunteers focus on **subjective parameters** (field perceptions, subjective state evaluations), **behavioural effects** (reaction time performance, memory and attention tests), **and neuro-physiological and psycho-physiological responses** (analysis of heart rate and cerebral electrical activity while awake or asleep or during attention tasks). Other aspects are also investigated, such as**circadian rhythms, neuro-hormonal, heamatological and immunological systems**.

Advantages of human studies

= logical complement of epidemiological studies (e.g., EHS: provocation studies)

- May help in finding an explanation of observed morbidity and mortality data
- May help in a better insight in pathophysiological working mechanisms
- Agents such as antioxidants can be applied to identify potential protective measures against exposure of polluents or mixtures

Limitations of human studies

- Ethical considerations may limit their application (e.g., studies on potential carcinogens)
- Ethical considerations prevent investigations on certain target populations (e.g., children)
- These studies are limited to acute exposures and effects
- These investigations can only be performed on a limited number of subjects
- Usually also quite expensive
- Usually a specific laboratory infrastructure is necessary which is not readily available (only in specialised laboratories)

Animal experiments: in vivo studies

It consists in exposing living animals (mice, rats, guinea-pigs, etc.) to electric and magnetic fields.

Exposure time is variable, and can last as long as the animal's life. Tests make it possible to determine if the fields have affected the embryonic development, growth, fertility, behaviour or physiology of the animal.

Advantages of animal experiments

Advantages usually the same as for clinical studies, but:

- A large number of animals can be studied (contrary to humans)
- Relatively cheap compared to clinical studies
- More invasive procedures can be used to investigate biological effects from exposure to electromagnetic fields
- Chronic exposure can be studied (e.g., life-time exposures of mice and rats)
- Specific animal models can be used (e.g., extra sensitive strains, genetically modified animals, …)
- ….

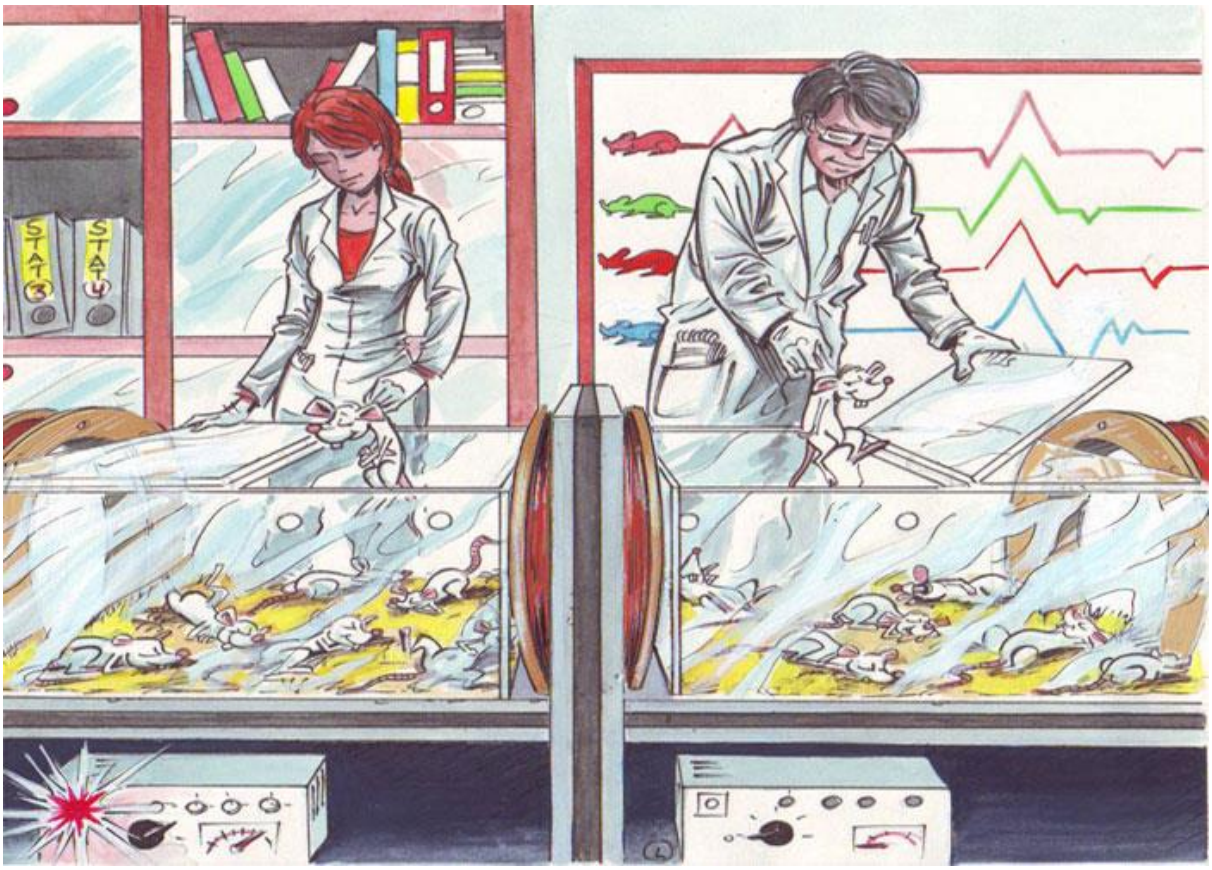
Limitations of animal experiments

It is indeed difficult to extrapolate the results of animal studies to the case of actual human exposure. Animals are not humans and the biological effects observed in the animal are obtained under very specific experimental conditions.

Here are some limitations that prevent results in animals to be automatically transposed in humans:

- Applied doses are not always equivalent between species
- Differences in life expectancy
- Differences in size
- Differences in diet
- Differences in genetic variability
- Differences in amount of antioxidants
- Ethical problems
- …

Extrapolation to humans can be very difficult. **A valid methodology** is of crucial importance!



1 A valid experiment - Exposed versus control groups

A crucial point in EMF experiments is to make sure that fields are actually the variable responsible of whatever effects tested. It means that two groups are needed: the first one, the exposed group, will be placed under EMF exposure, while the other, the control group, will be sham exposed. The only difference between both groups is the EMF exposure. All other parameters need to be accurately controlled. Researchers cannot avoid working with two groups in their labs. Control and exposed groups must be identical in all relevant ways except for the EMF exposure of the experimental group.

Studies that compared the results of their exposed group to the results of a control group found in the literature are not valid.

To avoid observer bias/error, all experiment stages should be made by the same scientist and, in any cases in double blind.

2 A valid experiment - Double blind

Double blind in animal studies means that neither the person that breeds animals, nor the person that takes a blood sample, for example, have information on which group is really exposed or not. Each group needs to be treated in the same manner. The purpose of a double blind experiment in animals is to avoid observer bias and even some placebo effects in animals.

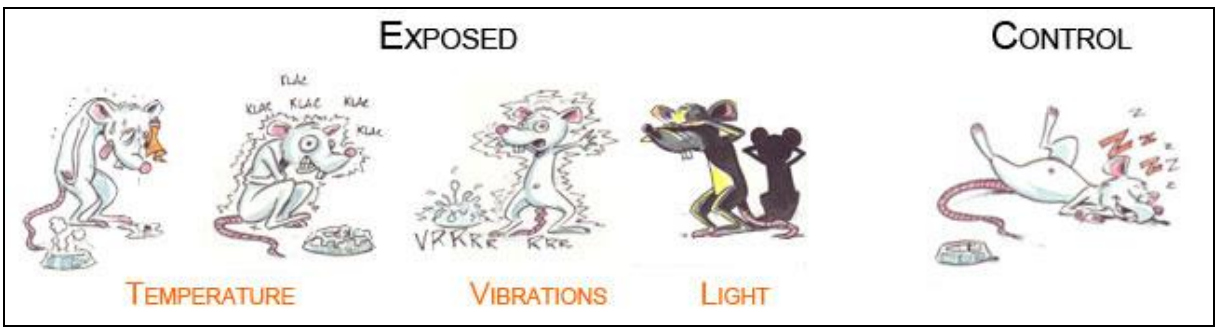
Here is a caricature of a study that not reaches the double blind objective. It is not valid.

"Contrarily to the picture, each group needs to receive the same treatment."

3 A valid experiment - Same conditions

It is obvious that each group also needs to be physically in the same conditions, but it is not always the case: vibrations, noise, higher or lower temperature, light… can disturb the life of the exposed group.

Let us think for example of a power generator that should be noisy or even slightly vibrating.



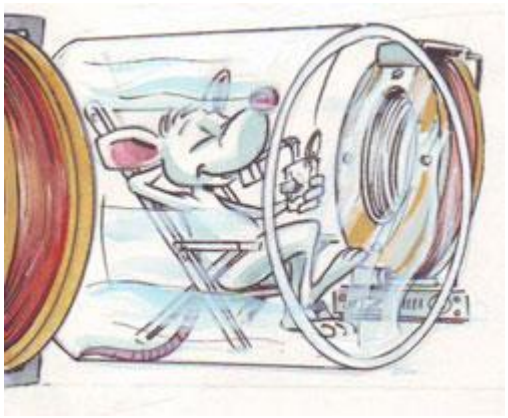
4 A valid experiment - Exposure system

It is important that researchers have accurate information on animal exposure. Unfortunately, studies are too often deficient with respect to the exposure assessment.

"*Rats sleeping just above the coil are over exposed compared to other rats."*

As intensity quickly decreases with distance, an animal could spend too much time just above the coil while another could be less exposed because of its movement in the

The best solution to avoid differences in intensity should be to place animals in closed tubes surroundings by coils.



Of course a huge cage seems stressless than this kind of confined tube. However, by experience, scientists know that it does not create more stress in animals.

Comparison with a control group in the same condition is compulsory.

Characteristics of exposure system are another flaw in lots of published scientific papers. Poor information is provided concerning the generator, the frequencies used and their harmonics, the signal (continued or pulsed) …

5 A valid experiment - Animal models

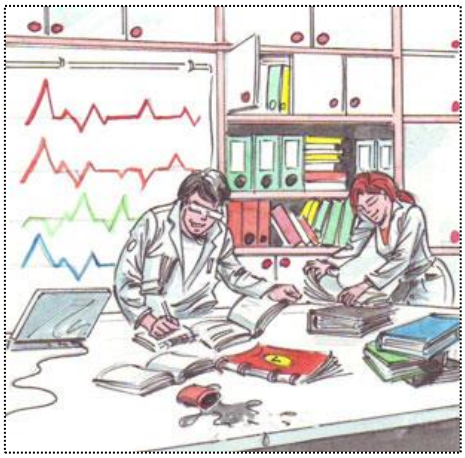
Mice, rats or guinea pigs are classical animals in labs. According to the aim of the studies, they will be normal or transgenic. Transgenic means that animals are genetically modified to become more susceptible to certain pathologies, skin cancer for example.

"Because they are easily bred, it should be far simple to work for example on insects, but they are too different from us than mice or rats."

6 A valid experiment - Study replications

According to the difficulty of having perfect experimental conditions and the fact that all parameters cannot be controlled, results of one study mean nothing.

"Results of a single study are not sufficient to validate a theory and to break open a bottle of champagne."



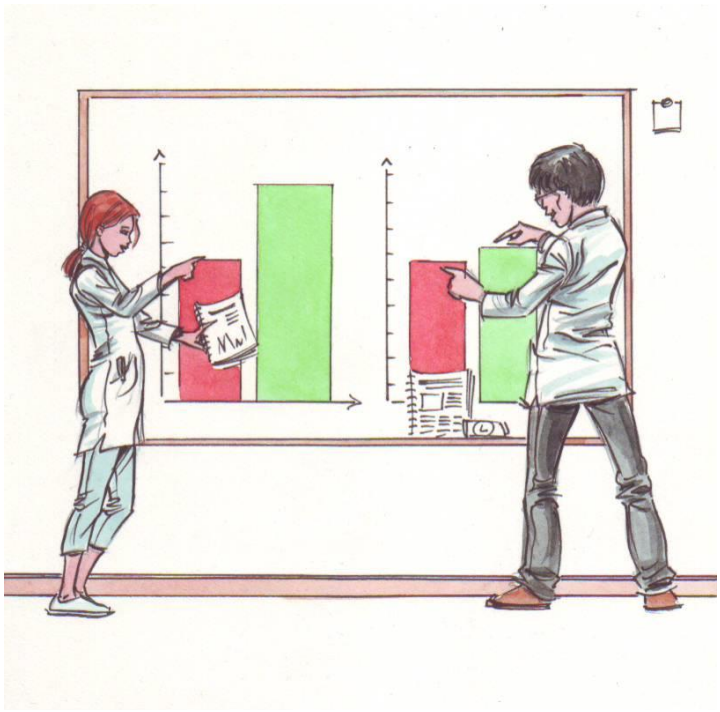
"It is compulsory to replicate a study and to compare with results of other laboratories. "

According to the World Health Organization, a single study is accurate to formulate a hypothesis, not to validate a theory.

7 A valid experiment - Statistical analysis

Statistical analyses are an important part of a research. Results need to be analysed with cautious. Each step requires to be carefully defined: number of animals in each group, parameters to be evaluated, statistical tests… According to the group tested (size and characteristics) and the parameters taken into account, the statistical analysis will be different.

In some published studies, statistical analyses are difficult to interpret as details are not often provided.



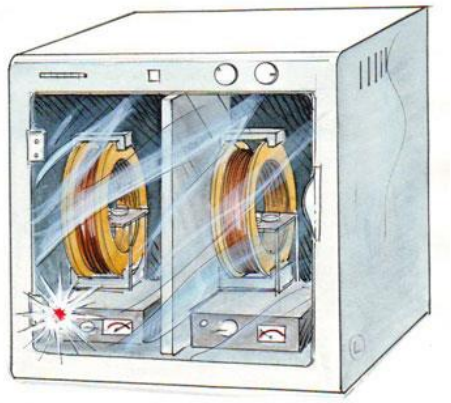
"Statistical analysis is a full-time job: only looking figures is not enough. Data need to be thoroughly analysed. Better not to play at being God!"

Moreover, let us remind that we can make statistics lean towards what we want it to lean towards. Statistical analyses always provide values. Common sense is crucial to evaluate their accuracies, before going further.

The interpretation of these values is another potential flaw. Researchers need to keep in mind the results of previous studies, the characteristics of their methodology… in order to brightly discuss the results.

Cells experiments: in vitro studies

In vitro studies consist in subjecting cells or tissues to low frequency electric and magnetic fields. The objective of *in vitro* studies is to be able to determine the potential influences of such fields, and to isolate them from other types of influences. However, they also have a major disadvantage: cells or tissues are removed from their natural environment, thereby eliminating the interaction and protection mechanisms otherwise available from the donor organism. Moreover, the fields used are generally stronger than the fields to which the population or workers are exposed. This can result in effects that do not exist with low field values.



It should also be emphasised that a modification that has occurred on a cellular level during tests does not mean that the whole organism would experience the same effects.

Note:
DNA damage => possibly genotoxic in humans
DNA damage ***in vitro*** => possibly but not necessary
DNA damage ***in vivo***

Advantages of in vitro studies

- Especially important to investigate and identify cellular/molecular working mechanisms:
  - You know exactly what you are doing
  - Your work can be very specific and detailed e.g., Investigations of cell division failures by looking at mitotic spindle apparatus or particular DNA studies, 'omics'…
- Fast (fast screening): negative ***in vitro*** = negative ***in vivo***
- Relatively inexpensive
- Often predictive of a real hazard or risk (e.g., DNA damage)
- High throughput screening:
  - ex: VITOTOX-test (see further in PubMed)
  - "Omics" (microarray technology, http://www.ncbi.nlm.nih.gov/pubmed/20809503)
  - Specific cell lines (lung or skin epithelial cells, white blood cells, hepatocytes….)

Limitations of in vitro studies

- Cells are treated outside their normal 'environment' (no surrounding tissues, no blood supply, no normal supply of nutrients, …)
- ***in vivo*** exposures cannot easily be mimiced
  (Metabolisation can be simulated by addition of specific chemical agents)
  => Enhanced credibility when same effects are also demonstrated *in vivo*.

1 In vitro studies: a valid experiment

Importance of the following points (further information in *in vivo* Studies):

- Exposed versus control groups
- Double blind
- Same experimental conditions
- Exposure system
- Cell lines: Tests in selected cell lines according to purpose and target:
  - Lung epithelial cells
  - Brain cells

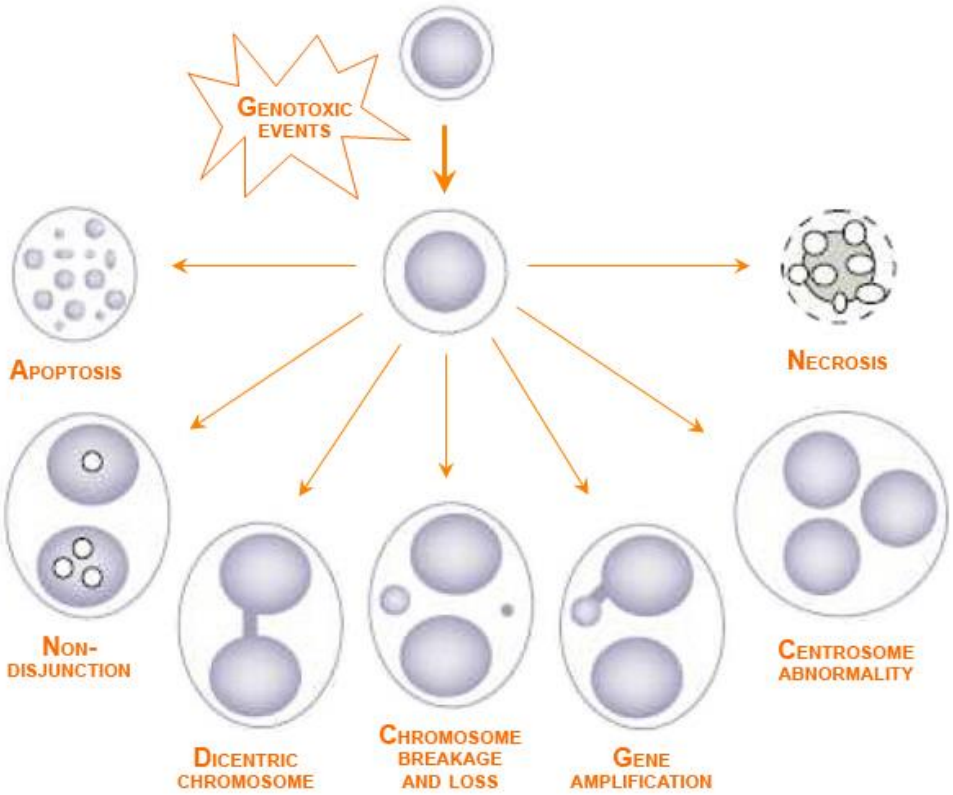
  - White blood cells
  - Liver cells
  - ….
- Study replications
- Statistical analysis

2 Examples of test on cells

Hundreds of tests are available to check the effects of an agent on cells. Here are two examples of tests: the cytome essay and the comet assay. Other tests are also described in BBEMG results - EMF effects on keratinocytes (Prof M Hinsenkamp).

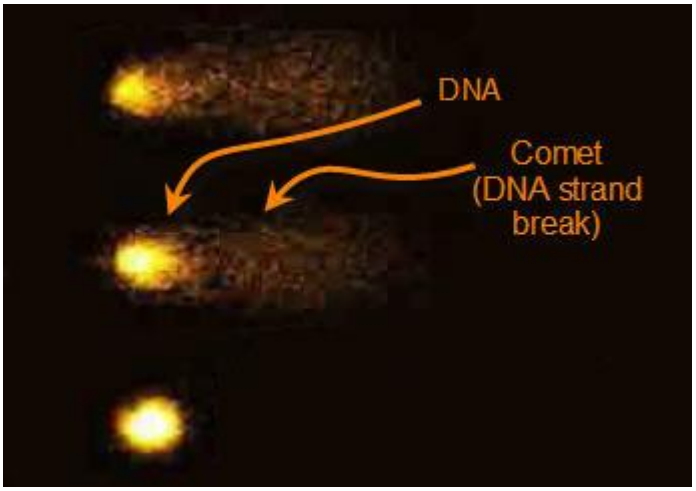
2.1 The cytome assay

The cytome assay can be considered as an extended "micronucleus test"; this means that cells are blocked in telophase, just before cell division. In this stage two main nuclei are present. In case of genotoxicity a number of abnormalities are present: micronuclei (broken chromosome fragments or lagging chromosomes are scored in the classical micronucleus test). Other morphological features give additional information: nuclear bridges (dicentric chromosomes), nuclear buds (gene amplification), trinuclear cells (centrosome abnormality). Also numerical chromosome aberrations (e.g., as a result of abnormal nuclear division = non disjuntion) can be scored using specific chromosome probes as well as apoptosis (programmed cell death) and necrosis (cell death).



Source: Fenech M. (2002) Chromosomal biomarkers of genomic instability relevant to cancer. Drug Discovery Today, 7, 1129-1136.

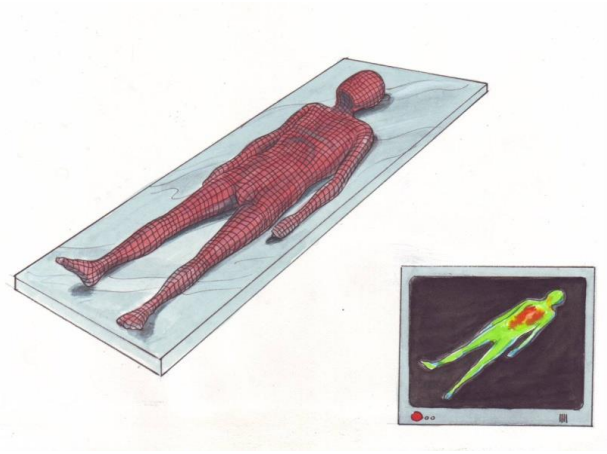
2.2 The single cell gel electrophoresis assay or Comet test



In the comet assay DNA from individual cells is embedded in agarose (gel) on a microscope slide and subjected to electrophoresis (electric current). When DNA is damaged, broken fragments migrate in the gel towards the positive pole. A comet-like structure is formed. The length and the intensity of the tail can be measured. Undamaged DNA has no (or very short) tails, the tail lenght is proportional to the damage.

Modelling

Mathematical modelling consists in building a mathematical representation of reality that attempts to explain the behaviour of some aspect of it, based on simplifying assumptions (hypotheses). The mathematical representation usually consists in a set of variables and a set of equations that establish relationships between these variables. The mathematical model can serve several purposes: answer a variety of what-if questions, understand the relationships between variables, extrapolate past data to derive meaning, etc. Models are typically used when it is either impossible or impractical to create experimental conditions in which scientists can directly measure outcomes. However, even when experiments are possible, obtaining a good mathematical model is usually very interesting, as it can provide insights into the internal workings of a system that direct measurements cannot.



In EMF research, a large effort is underway to construct mathematical models to calculate electric and magnetic fields generated by electrical devices (powerlines, transformers, engines, electronic circuits, furnaces...). Recently, researchers have been trying to extend such models to compute electromagnetic fields inside living organisms (from the cellular level all the way to the whole human body). Such models of the human body cannot usually be solved using a “pencil and paper” approach: they require the use of computers, which break the body into many simple geometrical shapes (for example little cubes), in which mathematical equations are solved.

Modelling is of major interest in defining guidelines and recommendations for limiting the exposure of the public to electromagnetic fields. For example, recent guidelines intend to avoid internal electric fields greater than 0.02 V/m for the public and 0.1V/m for workers (see Standards). Modelling allows to predict the magnitude of such internal electric fields in full-size phantom models or partial body models exposed to various external sources (magnetic fields, electric fields, contact currents...). Modelling is also pertinent in in vitro and in vivo studies to accurately assess the distribution of fields in cells and animals, according to the exposure system. It is a principal tool in assessing the biological dose resulting from EMF exposure.

Advantages of modelling studies

- Easily reproducible virtual “experiments”
- Cheaper than laboratory experiments
- Ability to test many variations
- Non invasive

Limitations of modelling studies

- Potentially wrong if based on bad simplifying hypotheses
- Potentially wrong if bad input data

The first limitation must be addressed by validating the mathematical model with repeatable laboratory experiments. Lack of agreement between theoretical mathematical models and experimental measurements often leads to important advances as better theories are developed. To try to mitigate the second limitation, probabilistic (stochastic) mathematical models are developed, which analyse the sensitivity of results with respect to uncertainties on the input data.