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Review Article

Quantitative Shifts of Electrogenic Metals in Epidermal Cells in the Setting of Oxidative/Nitrosative Stress – What Could They Stand For?

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Abstract

The paper informs about the specific shifts in metal-ligand homeostasis (MLH) of epidermic cells (hair) in Chernobyl accident liquidators, which are viewed by the authors as biomarkers of oxidative/nitrosative stress. The detected (by EPR-analysis) relationships between NO-production and MLH quantitative shifts can be indicative of the possible participation of nitric oxide in generation of cell electric potential.

In recent years, the method of quantitative spectrometry of bio-substrates such as human hair has become very popular in determination of person's elemental status. The suitability of the method for large-scale studies, its non-invasive sampling procedure, sample transportability and storability under normal conditions – are all factors that have attracted the attention of investigators. However, in recent publications on the topic questions about the relationship between elemental hair composition and the total body mineral content has been raised and requires special discussion.

First, according to atomic emission spectrometry data, concentration values of chemical elements (including but not limited to metals), which are contained in hair, show expressed individual variation: Coefficient of Variation (CV) averages 125.5±17.5% [1]. This very fact suggests that the observed shifts may be caused not nearly by 'hypo- or hyperelementosis' but by *redistribution* of chemical elements mediated by intra- and

extracellular regulators of transmembrane mineral traffic which has practically no effect on the total body elemental composition.

Second, evaluation of the elemental status of the cutaneous appendages (as part of the excretory system) must be very accurate when diagnosing impairments in metal-ligand homeostasis (MLH) at the level of the body as a whole. Such evaluation is required in cases when the object of study is an excretory product (e.g., urine). Ambiguity in interpretation of results of elemental analyses of biotic substrates of the excretory system (sweat, urine, expired air, epidermis and its appendages) may appear in case of failures in the systems responsible for the 'containment' of essential metals in the body (e.g., heme Fe). As is known, a single molecule of free (unbound) hemoglobin could easily pass the renal filter and show up in urine (hemoglobinuria). However, it does not happen normally due to the fact that hemoglobin is bound by a molecule of transfer protein, i.e. haptoglobin. The newly formed complex of 'hemoglobin-haptoglobin', because of its sizes, is no longer capable of penetrating through the basal membrane pores, thereby retaining a heme iron pool for resynthesis of hemecontaining proteins.

Under chronic (and often latent) hemolysis, hemoglobin iron may escape from the body with urine in the form of hemosiderin (lead intoxication, Marchiafava-Micheli disease). But can it be diagnosed by detecting an overall increased Fe level in the body? Certainly not! One can rather speak about an inevitable reduction in the total ferrum pool (due to continuous hemosiderinuria).

Similar examples may be given with regard to other metals as well. But does it mean that hair is not a very 'reliable' substrate for MLH evaluation? And though an unequivocal and substantiated answer to this question will take time and further studies, it is hardly reasonable to disregard such MLH evaluation in the hair solely on this ground.

To be objective, it is not the matter of 'trust' in the biosubstrate but the invalid application of extrapolations of data of trace elemental analysis of hair to the elemental content and distribution in the whole body. In other words, the problem is not with the substrate but with interpretation of MLH variations in epidermal cells meter mined by spectrometry.

There are many factors to be considered in determining the most probable causes of quantitative shifts in MLH. Their distinguishing feature is the capacity of activation or deactivation (up to a total block) of ionic channels – hydrous pores of transmembrane proteins, which are in charge of metals transfer. Depending on the mode of activation, the ion channels are classified into stretch-activated, voltage-activated and ligand-activated. Activation of ligand-activated channels may take place due to redox-modification of thiol groups of cysteine in the molecule of proteins-transporters. Among the latter ones is the P-type ATPases superfamily, which ensures the transportation of not just electrogenic (Ca, Na, K) but also heavy metals (Cd, Zn, Pb, Cu, Co, Ag) [2-4].

Active oxygen species (AOS) and active nitrogen species (ANS), which are constantly formed in cells: superoxide anion radical ($O_2^{\cdot-}$), nitric oxide (NO), and peroxynitrite ($NOOO^-$), fulfil the function of redox-modifiers of cysteine residues in the molecule of membrane pumps (oxidation with formation of disulphide bonds, S-nitrosylation). Having said this, one cannot exclude that increased production of $O_2^{\cdot-}$ and NO (oxidative/nitrosative stress) which may cause further activation of P-type ATPases (maybe, due to the involvement of more protein molecules into the process of transmembrane transfer and/or participation of more aggressive ANS as redox-modifiers). Therefore, in the setting of oxidative/nitrosative stress, we may expect quantitative shifts in intracellular concentrations of not just electrogenic but also heavy metals (transfer of the latter is affected by $P_{1B\text{-type}}$-pump from the superfamily of ATPases).

The plausibility of such expectations may be confirmed (or refuted) through investigation of MLH in the setting of oxidative/nitrosative stress, e.g., in the Chernobyl accident liquidators. Experience has shown that the main distinguishing feature of the biochemical processes in the bodies of Chernobyl liquidators is an increased (compared to the norm) activity of oxygen [5] and nitrogen [6] radicals or chronic oxidative/nitrosative stress, which may relate directly to the events taking place in MLH.

By the method of quantitative EPR-spectroscopy using an *in vitro* complex of Fe^{2+} with diethyl-dithiocarbamate (DETC) as a spin trap of NO, which forms with it paramagnetic mononitrosyl ferrum complexes with DETC (MNFC-DETC), we managed to assess the level of NO and Fe^{3+} complexes (EPR signal g=4.3) in such a biotic substrate as epidermis appendage (hair) [6]. The levels of NO and Fe^{3+} complexes (EPR signal g=4.3) in the hair, as assessed by the EPR method, in the accident liquidators (n=45) significantly ($p<0.05$) exceeded the normal values (25.9±1.8 *vs* 20.7±2.5 and 0.11±0.01 *vs* 0.07±0.004 *resp.*). A higher, versus the norm, level of not just NO, which is determined by the quantity of MNFC-DETC, but also Fe^{3+} complexes (g=4.3) in the Chernobyl accident liquidators, gives ground to consider both of these indicators as nitrosative stress discriminators.

At the Biotic Medicine Centre (Moscow), mineral composition of hair was analyzed by the atomic emission spectrometry method using the Optima 2000 DV Device in 947 healthy subjects (238 males and 709 females aged 2 to 86) and 954 liquidators of the accident at the Chernobyl Atomic Electric Power Station – Moscow residents (213 females and 741 males aged 37 to 82) [7]. We performed verification of the normal distribution hypothesis by the Jarque-Bera Test [8] and the Kolmogorov-Smirnov Test [9]. As a result of such verification, we managed to disprove, with a higher probability, the hypothesis of normal distribution of chemical elements [1]. Therefore, we used alternative approaches (*bootstrap methods*), not requiring normal distribution of *a priori* assembly [10]. When detecting possible gender-based distinctions in the mineral composition of hair, we found that the level of calcium (Ca) had depended on the gender. Therefore, Ca concentration values were registered for males and females separately.

Furthermore, in overall the spectrometry data in the Chernobyl accident liquidators (n=954) and healthy subjects (n=947) determined the linear correlation ratio by the $\boldsymbol{r}$ coefficient (Pearson) between the concentration values of potassium (K) and zinc (Zn). As a result, another criterion of distinction has been determined: in the Chernobyl accident liquidators, $\boldsymbol{r}_{K\text{-}Zn}$ (-0.42; $p<0.05$) turned out to be by 50% greater than in healthy subjects ($\boldsymbol{r}_{K\text{-}Zn}$= -0.28; $p<0.05$).

In order to determine how homogeneous the studied groups were in regards to the value of $r_{K\text{-}Zn}$, we used the method of selective sampling (which we had put forward earlier) of persons with maximum and close to zero values of the ***r*** coefficient from the general totality [11].

It was found that the absolute majority of the accident liquidators (88%) showed a negative and significant K-Zn correlation (in 205 persons $r_{K\text{-}Zn}$ = -0.62; p<0.05; in 634 persons $r_{K\text{-}Zn}$ =-0.41; p<0.05). In 12% of the Chernobyl accident liquidators (115 persons), it was not detected (***r*** = -0.03). The K-Zn correlation was absent (***r*** = -0.01) in 253 healthy subjects (26.7%), feebly marked ($r_{K\text{-}Zn}$ = -0.22; p<0.05) in 523 persons (55.2%) and obviously marked ($r_{K\text{-}Zn}$= -0.43; p<0.05) in 171 persons (18.1%) only.

Symptomatically, the strongly noted positive correlation between K and Na, which amounted to $r_{K\text{-}Na}$=0.64 for the whole group of healthy subjects (n=947), remained practically the same (0.64; 0.71; 0.53; *resp*.) at different $r_{K\text{-}Zn}$. This can be explained by the stable (failure-free) operation of a K/Na-pump in healthy subjects. Just as close (***r*** =0.64-0.75) was the K-Na connection in the absolute majority of the Chernobyl accident liquidators (in the whole group $r_{K\text{-}Na}$=0.71) but visibly less close in the subgroup with $r_{K\text{-}Zn}$ = -0.03 ($r_{K\text{-}Na}$=0.31).

The concentration values of Zn and electrogenic metals (K, Na, Ca) in the accident liquidators' epidermis had significant ($p<0.05$) distinctions compared to the control group, and in the Chernobyl accident liquidators they were as follows: Zn=162.5<**165.8**<169; K=365.8<**394.8**<422.4; Na=757.5<**822.3**<892.4; $Ca_{(fem.)}$=832.0<**927.4**<1032.8. In healthy subjects: Zn=181.5<**185.2**<189.3; K=277.4<**317.7**<361.1; Na=427.9<**480.9**<542.9; $Ca_{(fem.)}$=1348.1<**1439.6**<1528.4 (the boldface characters are the medium value (M) in *mcg/g*; the normal type – the confidence limits – *the bootstrap-method*).

The analysis of MLH of electrogenic metals and zinc in epidermal cells of healthy subjects at the extreme values of $r_{K\text{-}Zn}$ (-0.01 и -0.43) has shown that shifts in MLH of healthy persons with $r_{K\text{-}Zn}$= -0.43 (*vs.* $r_{K\text{-}Zn}$= -0.01) are, by their directionality number of cases and by their magnitude, similar to those of the total group of the Chernobyl accident liquidators (n=954), when compared with the total group of controls (n=947). One can draw a conclusion, (which seems obvious although requiring verification) about the presence of oxidative/nitrosative stress of non-radiation nature in healthy subjects with $r_{K\text{-}Zn}$= -0.43.

Similar changes in concentration values of electrogenic metals and zinc dependent on the value of $|r|_{K\text{-}Zn}$ have been also detected in the Chernobyl accident liquidators.

If the negative correlation ratio K-Zn in the setting of oxidative/nitrosative stress really reflects the activity of the superfamily of membrane ATPases (which is indirectly suggested by the spectrometry results), the revealed 'sensitivity' of electrogenic metals to increased production of AOS and ANS, which, alongside with the growth of $|r|_{K\text{-}Zn}$, is accompanied by observable shifts in intracellular level of Ca^{2+}, K^+, Na^+, must inevitably lead to changes in the electric potential (EP) of the cell.

Moreover, we have grounds to suggest that such changes may be of oscillatory nature due to the cyclic variation of the level of NO and nitrosonium ions (NO^+) in cells and tissues. As it has been shown [12], NO, Fe^{2+} ions and low-molecular thiols are capable of forming a self-sustainable, self-regulating chemical system with constantly appearing S-nitrosothiols and dinitrosyl ferrum complexes (DNFC) with thiol-containing ligands. Their interconversion ensures oscillatory alteration in the level of the compounds and the related periodic oscillations in the content of NO, NO^+, ferrous iron and thiols, which do not constitute S-nitrosothiols (DNFC), by type of the Belousov-Zhabotinsky reaction. Cyclic alteration of the nitrosonium ion level capable of thiol S-nitrosating [13] may synchronically (in oscillative mode) change the activity of membrane ATPases, which contain thiol groups, thereby causing oscillation in the level of electrogenic metals and the oscillation-related changes in the EP of cells and tissues.

Thus, the significance of the NO^+-producing system proves to be considerably more influential. This applies primarily to the cells, whose main function is directly related to the EP generation (neurons, pacemaker cells, cardiomyocytes, vascular and intestinal myocytes, etc.).

The modern concept of the nature of cardiac automatism is based on the probable existence of two synchronically operating EP oscillators in the pacemaker cells and cardiomyocytes: in the membrane of sarcoplasmic reticulum (SR), the so-called *'calcium clock'*, and in the outer membrane – the *'membrane clock'*. Transmembrane Ca^{2+} migrations, causing changes in the EP, take place under active participation of ATP-dependent calcium pumps, which operate, according to the researchers, on an automatic mode [14,15]. The pacemaker's role in this process is assigned to 'calcium clock', whose relationship with the 'membrane clock' is based on the subordination principle. One of the reasons in favour of such role assignment is based on the fact that full cut off of the 'membrane clock' does not affect the operation of the 'calcium clock' [16], whereas the shutoff of the latter means the stoppage of the 'membrane clock', which, however, was 'restarted' by means of an extra quantity of Ca^{2+} [17].

Calcium 'sparks' (i.e. local and periodic increase in Ca^{2+} concentration at various locations of cardiomyocyte myoplasm), which are detected by confocal microscopy, are viewed as an illustration of the 'calcium clock' operation. The cyclic changes of the NO level, in coincidence with Ca^{2+}, in the myocardium during its contractions are associated with the oscillations of the Ca^{2+} level in the myoplasm [18].

Notwithstanding the logic of such reasoning, one can hardly ignore doubts regarding the correctness of the choice of a pacemaker in the system of oscillators: *'NO-oscillator'→ 'calcium clock'→ 'membrane clock',* where the 'calcium clock' plays the role of a pacemaker (?).

The events taking place in epidermal cells in the setting of oxidative/nitrosative stress, when changes in the content of electrogenic metals (including Ca) appear to be the consequence rather than the cause of pro-oxidant shifts, justify the choice of NO^+ (as a pacemaker of the aforesaid oscillators), which is generated through autocatalysis. This is further evidenced by the

universality of the RS-NO↔DNFC self-oscillating system, which (unlike sarcoplasmic reticulum) may be found practically in all cells.

Why is it so important to make the right choice of a pacemaker in the presence of several oscillators functioning concurrently?

The fact is that cyclicism is an inherent feature of natural phenomena. Of special importance in living systems, however, is not only the mere existence of cyclic processes (quite a few of those have already been detected, including chemical interactions) but also their organization in time and space, their synchronizing and self-regulating ability. Normal functioning of cells and the whole body depends on the 'correct' operation of one or several oscillators united into an auto-oscillating system by the subordination principle, as well as on the synchronous interaction of a number of self-oscillating systems. The example of cardiac oscillatory systems, whose co-subordination forms the following sequence: *sinus node myocytes (pacemaker) → atrial myocytes → ventricular myocytes* demonstrates how a faithful representation of interactions between such oscillators cans facilitate diagnostics of failures in their synchronous operation.

As regards electrogenic metals, their interactions in cardiomyocyte may be confined to the following. NO^+-oscillations, which cause synchronous (under oscillating conditions) S-nitrosation of thiols in ATP-ase molecules and the rhyanodine receptor RyR2, induce (also under self-oscillating conditions) transmembrane traffic of electrogenic metals (and, above all, Ca^{2+}), thereby resulting in action potential (AP), muscular contraction and muscle relaxation.

In this case, the working rhythm of 'calcium' and 'membrane clock' will depend on synchronous ('bursting') appearance in the cell of *sufficient* quantity of NO, (NO^+) for S-nitrosation of membrane pumps and *adequate* transmembrane transfer of Ca^{2+}. The main criterion of '*sufficiency*' of the working molecules (NO, NO^+) is high synchronization (at least, not lower than *critical*) of individual {RS-NO↔DNFC} systems' oscillations.

The term 'critical synchronization' needs explanation. It was borrowed from the currently existing *self-organized criticality* theory describing excitation processes and information transfer in neural networks of the brain [19]. According to that experimentally verified theory, brain operation may be compared to the functioning of a complex dynamic system, which may exist in any the following three working states: a) subcritical, b) critical, and c) supercritical. The theory authors explain the necessity of the system's critical condition by the fact that information received by the brain (excitation) must be accessible to all of its divisions at once and, therefore, capable of propagating for relatively long distances. In other words, it is essential that changes in input neurons' parameters be transferred to output neurons, which are located at quite a distance. This prerequisite may only be met if the system is in a critical or supercritical state. The main criterion, which allows assessing this or that status of a system, is the branching parameter σ.

If an input neuron's excitation is transferred, on average, to less than one neighboring neuron ($\sigma < 1$), the state is defined as 'subcritical'. In case of critical state, the excitation of input neuron is perceived, on average, by one adjacent neuron ($\sigma = 1$). Supercritical state of a neural network is characterized by excitation transfer from an input neuron, on average, to more than one adjacent neuron ($\sigma > 1$).

The main postulates of the critical states theory may be used for explanation of the working principle of NO^+-generating {RS–NO↔ DNFC}–systems, the only difference being that no excitation transfer, like in neural networks, takes place here but adjustment of self-oscillating systems' rhythm in the setting of their weak interaction (synchronization). Such adjustment (although, not in electromagnetic but mechanical self-oscillating systems) is demonstrated by C. Huygens's classical experiment: if two pendulum clocks are hung on the same wall, the pendulums' motion will become synchronous after a while. Nothing like that happens when the clocks are hung on different walls.

Understandably, the probability of spontaneous synchronization, given weak interaction of the systems, depends directly on their distribution density in a definite volume (in this case – on the concentration of {RS–NO ↔ DNFC}– oscillators in cytosol). Therefore, when a cell lacks the main components of self-oscillating systems (above all, NO and Fe^{2+}), their synchronous interaction level may turn out to be subcritical ($\sigma<1$). An increase in concentration of {RS–NO↔DNFC} by replenishing the deficient NO and/or Fe^{2+} must increase synchronization to, at least, the critical level.

As an example of subcritical synchronization level of the {NO↔DNFC}–systems one may mention local (as a consequence of atherosclerotic changes in vessel wall) impairment of blood flow in coronary vessels at the height of an anginal attack, when the closely interconnected components of vascular motion (muscular contraction and muscle relaxation) turn out to be inadequate. The problem is solved by increasing the synchronization degree of the operating oscillating systems NO↔DNFC to the critical level ($\sigma = 1$) using the NO donator (nitroglycerine). The occurrence of full-fledged synchronic contractions and, just as important, relaxations of vascular smooth muscle cells (VSMCs), or, in other words, restoration of vascular peristalsis, will generally normalize perfusion of the stunned section of myocardium.

Interestingly, nitrates' inotropic effect on cardiomyocytes is well-known, whereas the antianginal action of these agents under ischemic heart disease (IHD) is readily associated by many clinicians with vasodilatation only. Although normal perfusion of cardiac muscle equally requires both relaxation and contraction of VSMCs (it is essential though that both components of vascular peristalsis be completely adequate and synchronized).

Is it possible under angina pectoris to restore full-fledged motion of coronary vessels and cut short the anginal syndrome without any additional (e.g., at the expense of nitrates) nitroxide introduction? This question was answered in the affirmative over a decade ago, when it became possible to apply (for clinical purposes) electromagnetic oscillations of a terahertz band (100 GHz to 10 THz) on a frequency of nitroxide's molecular spectrum [20]. A clear clinical effect (pain management) was observed alongside with an increase in the NO 'ability' due to prolongation of its life.

Nitrate overdose and the related sharp reduction of vascular tone, as well as hyper production of NO (the cause of collaptoid

states under all kinds of shocks), are, most probably, suggestive of a supercritical (σ > 1) level of synchronization. Not by coincidence, administration of methylene blue solution, which binds NO surplus when coupling state of shock, helps restore normal vessel motion and tone, perhaps due to restoration of the critical (or close to critical) level of synchronization. Whereas functioning of the {RS–NO↔ DNFC}–oscillators close to the critical synchronization level (σ = 1) ensures normal muscular contraction/relaxation not only of cardiomyocytes but also myocytes of blood vessels. At the same time, de-synchronization of the {NO↔ DNFC}–oscillators (σ < 1) may also be caused by Fe^{2+} deficiency. A clinical analogue of the situation is the sideropenic syndrome, which fully reveals the signs of nitroxide's functional inability.

The variety of clinical symptoms of iron deficiency (ID): from muscular weakness and apoptosis activation in regenerating tissues to cognitive disorders and all the scope of 'exotic' symptoms (pica chlorotica), may be explained by *functional inability)* of nitroxide, whose participation, as evidenced by many studies, is vitally important for prevention of such disorders under normal physiological conditions. Most probably, the cause of NO inability under ID lies not in its reduced production in the cell (although one cannot fully exclude such a possibility) but in de-synchronization of {NO↔DNFC}–oscillators (subcritical level of synchronization), which noticeably reduces long-term performance of NO.

Using the Electronic Paramagnetic Resonance (EPR) method, we quantified the NO-radical level in cells with ID in a group of undergraduates [21]. The EPR-analysis results showed significant reduction under the ID of the NO-radical level in the epidermis as compared with a control group: 15.7±1.2 *vs* 20.7±2.5 *($p<0.05$)* respectively. A correlation analysis (Pearson) was performed to measure the correlation ratio between the intensity of the EPR-signal of nitroxide and the severity of Fe-deficiency; it showed a positive linear relationship between the level of serum ferritin (criterion of adequate iron supply) and the height of NO-spike on a spectrogram. The correlation ratio ***r*** equaled 0.49 ($p < 0.05$).

To that effect, the incidence of angina pectoris in Russia's population showed an explicit gender-based dependence: in men – 3.3%, in women – 7.05% (as a reminder, the ID is diagnosed mostly in women) [22].

Certainly, the heart is not the only organ capable of generating an action potential in an automatic mode. Such capability is also inherent to the majority of a hollow internal organ with a muscular wall. This can be confirmed by the presence of an independent metasympathetic nervous system in such organs, which ensures realization of action potential without any 'command regulation' on the part of *n.vagus* or sympathetic nerves.

In conclusion, I would like to add the following. It is known that a living cell (despite the kind of tissue it belongs to) generates the EP (Z-potential). Technically, however, it is not always possible to reveal the rhythmical (oscillatory) nature of the EP (e.g., in the brain neurons, cardiomyocytes or skeletal muscles). If we assume that under synchronous interaction of a number of oscillators, involved in the EP generation, the role of the *order parameter* belongs to the self-oscillating system RS-NO↔DNFC, then the oscillatory nature of the EP, which is generated by the cell, must possess universality and extend to all cell categories.

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