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Pathol Int (Letter to the Editor)

Non-thermal plasma as a simple ferroptosis inducer in cancer cells: a possible role of ferritin

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To the Editor:

A large number of cooperative projects are currently in progress between engineering and medical researchers worldwide. Plasma is the 4th condition of physical states out of the normal solid/liquid/gas phase and is a mixture of radicals, electrons, cations, anions and light [1]. Aurora and thunder are typical plasma in nature, and plasma is even more abundant in space as the sun itself is plasma. However, they are usually in extremely high temperature. Non-thermal plasma (NTP), called also as low-temperature plasma, cold plasma or non-equilibrium atmospheric plasma, only became available as a novel engineering device in the late 1990's, which emits plasma of near body-temperature. Since then, many researchers are studying its possible applications to medicine, including its use for wounds in the battlefields and as a cancer therapy [2].

"Plasma medical science innovation" has been a national project in Japan, which was designated as an innovative research area by the Ministry of Education, Culture, Sports, Science and Technology of the Japanese government. Masaru Hori (Plasma Nanotechnology Research Center, Nagoya University) headed as the leader of this research group since 2012 to the present. Nagoya University has produced a machine that emits NTP of the highest electron density (1.6×10^{16} cm^{-3}) as far as we know [3]. We have thus far characterized the biological effects of NTP and found that direct NTP exposure can confer oxidative stress of specified intensity precisely to the designated location [4]. We have applied most of the possible preexisting methods [5], including measurement of conjugated diens, thiobarbituric acid-reactive substances, 4-hydroxy-2-nonenal-modified proteins, acrolein-modified proteins, 8-hydroxy-2'-deoxyguanosine and also cyclobutane pyrimidine dimers, which were all significantly and dose-dependently increased after an exposure of NTP. Electron spin resonance (ESR) using spin-trapping agents, which is a physical method to identify free radicals,

showed that direct NTP exposure mainly produced hydroxyl radicals [4].

Hydroxyl radicals are chemically produced through the Fenton reaction both *in vitro* and *in vivo*. In most cases, iron works as a catalyst: $Fe(II) + H_2O_2 \rightarrow Fe(III) + {\cdot}OH + OH^-$. Iron is the most abundant heavy metal in humans and 2.5-4 g is present in the whole body. Whereas its deficiency causes anemia, its excess is a risk for cancer [1, 6]. We demonstrated that local iron excess is the major pathogenesis of asbestos-induced mesothelical carcinogenesis [7]. Thus, we have used malignant mesothelioma cells for the NTP exposure, which were more sensitive than fibroblasts. Furthermore, mesothelioma cell death was non-apoptotic, proportionally iron-dependent and with increased catalytic Fe(II) in the cytoplasm [8], which thus falls into the category of ferroptosis. Ferroptosis is a recently defined type of regulated necrosis, where iron-dependent lipid peroxidation in phospholipids and the antagonizing cystine/glutamate antiporter and glutathione peroxidase 4 are the key regulators [9]. Intriguingly, a redox-inactive iron chelator to cover all the 6 ligands of iron can prevent ferroptosis whereas Fe(II)-rich cancer cells are specifically killed by NTP [8].

Iron is also a nutrient for cells. No species on earth can live without iron [7]. Cancer cells accumulate iron through transferrin receptor and divalent metal transporter 1 (SLC11A2) for their proliferation, where catalytic Fe(II) is generally increased in cancer cells in comparison to non-tumorous cells [10]. Simultaneously cancer cells are under oxidative stress in comparison to their counterparts [7, 11]. These could be the Achilles' heel of cancer cells. Fe(III) is almost insoluble ($10^{-35.5}$ M) to water at neutral pH. Ferritin, a 440 kDa protein consisting of 24 units, is localized in the cytosol as iron storage and one molecule can harbor ~4,500 Fe(III) molecules as non-catalytic safe condition. In previous experiments on the use of NTP, several different molecular mechanisms have been suggested, including apoptosis in glioblastoma [12] and lysosome genesis/autophagy in mesothelioma [8]. We hypothesized that NTP-

induced destruction of the shell of ferritin and simultaneous reduction from Fe(III) to Fe(II) may be one of the novel molecular mechanisms.

Here we have performed an *in vitro* experiment, using ESR spin trapping [4], to evaluate whether NTP can reduce Fe(III) within ferritin to release Fe(II), by the use of Fenton reaction (**Fig. 1)**. We observed the generation of hydroxyl radicals with the combined use of ferritin (from equine spleen, Sigma-Aldrich), H_2O_2 and NTP exposure in the presence of a spin trapping agent, 5,5-dimethyl-1-pyrroline N-oxide (DMPO; Labotec, Tokyo), indicating that stored Fe(III) in ferritin was indeed reduced to free catalytic Fe(II) after release from ferritin. Therefore, in irradiated cells with NTP, certain portion of Fe(III), stored in ferritin, may be reduced to free catalytic Fe(II), initiating Fenton reaction to cause oxidative cell death, which further requires demonstration *in vivo* in the near future.

In summary, it is highly possible that we now obtained a handy method (NTP) to induce ferroptosis in cancer cells. Cells with excess iron are more susceptible to NTP in theory [7] and catalytic Fe(II) release from ferritin could be another molecular mechanism. Recently, we succeeded in killing only the iron-loaded epithelial cells in an ovarian endometriosis model as a preclinical study, where the epithelial cells are the targets of carcinogenesis [13]. This research area must be novel for most of the pathologists. Further studies are warranted because iron is probably the most fundamental element for the origin of life on earth.

Disclosure statement

None declared.

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Figure legends

Figure 1. Electron spin resonance (ESR) spin trapping demonstrates the release of catalytic Fe(II) from ferritin with non-thermal plasma exposure. In a 96-well plate, 93.75 μg of ferritin in 150 μl of 10 mM phosphate-buffered saline (PBS) was exposed to non-thermal plasma (NTP) as described [4] for the specified period (1, 3 and 5 min), to which PBS, DMPO (final 1:100 dilution) and H_2O_2 (final 100 mM) were added sequentially to a total volume of 300 μl. Immediately after mixing, a portion was pipetted into the ESR cell, followed by an ESR measurement for 2 min as described [4]. **, P<0.01; ***, P<0.001 *vs* 0 min with Student's *t*-test (N=3; means±SEM); ns, not statistically significant; star, Mn markers; filled circle, signal of hydroxyl radical as 1:2:2:1. Typical ESR records are shown. Y = 0.047 X + 0.23 (regression analysis; r = 0.80, P = 0.0019).

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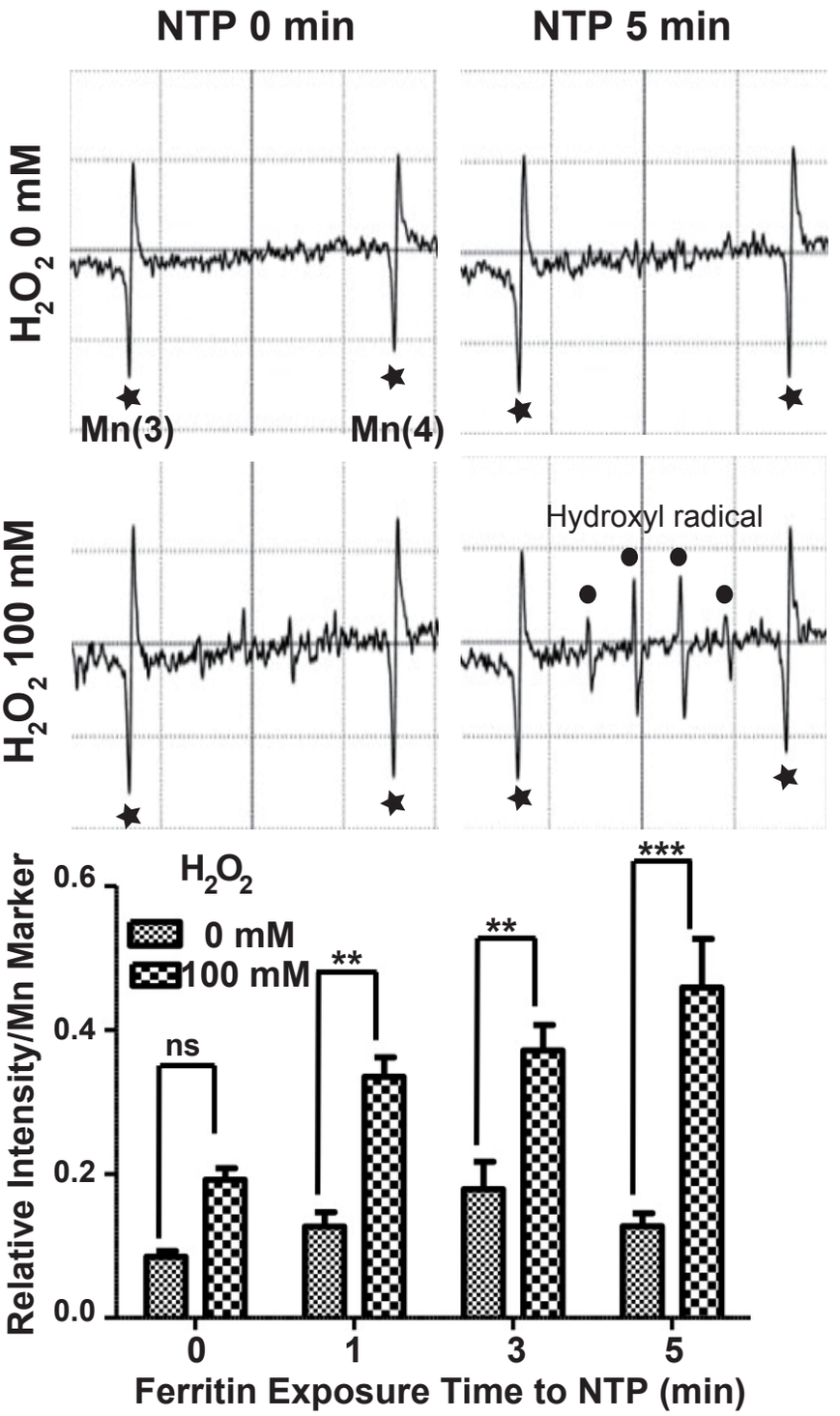
NTP 0 min
NTP 5 min
H_2O_2 0 mM
Mn(3)
Mn(4)
H_2O_2 100 mM
Hydroxyl radical
H_2O_2
0 mM
100 mM
ns
**
**
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Relative Intensity/Mn Marker
0.0
0.2
0.4
0.6
0
1
3
5
Ferritin Exposure Time to NTP (min)

Figure 1