Journal of Sciences, Islamic Republic of Iran 32(1): 15 - 28 (2021) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

Review Article

The Potential of 5-aminolevulinic Acid as a Contrast Agent in MR Imaging: Challenges and Opportunities

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Received: 22 October 2020 / Revised: 25 November 2020 / Accepted: 8 December 2020

Abstract

Nowadays, 5-aminolevulinic acid (5-ALA) plays an influential role in the detection of malignant tumors as a photodynamic diagnosis tool. Nevertheless, an outlook regarding 5-ALA applicability in magnetic resonance imaging (MRI) has recently emerged. Many studies confirmed the impact of 5-ALA on promoting intracellular Heme synthesis and iron metabolism, which support the capability of 5-ALA in MRI owing to the susceptibility effect of iron. Therefore, concerning the high safety and high affinity of 5-ALA to tumor cells, 5-ALA-based MRI could be an intriguing method for malignant foci identification.

Keywords:5-aminolevulinic acid; Magnetic resonance imaging; Protoporphyrin IX; Cellular iron metabolism; Transferrin receptor.

Introduction

5-aminolevulinic acid (5-ALA) is an amino acid prodrug in the Heme synthesis pathway [1, 2]. The polymerization of eight 5-ALA leads to the fluorescent protoporphyrin IX (PpIX) synthesis, functioning as a direct biological precursor of Heme [3-5]. The exogenous 5-ALA administration results in selective accumulation of the PpIX in cancerous and precancerous lesions, allowing for discrimination between tumors and normal cells [6, 7]. So, 5-ALA has been approved using the US food and drug administration as a photodynamic diagnostic (PDD) tool for high-grade glioma resection [8]. Moreover, as an effective photosensitizer, higher accumulation of PpIX in malignant cells than normal cells has been employed for photodynamic therapy (PDT) of many types of cancers including lung, colon, gastric, breast, ovarian, brain, renal, melanoma, and prostate cancers [9]. PDD and PDT are two major applications of 5-ALA in medicine.

Several reports have studied the effect of exogenous 5-ALA on iron metabolism, confirming iron accumulation following exogenous 5-ALA administration [10, 11] The ability of 5-ALA to change the susceptibility effect associated with iron accumulation in the Heme signaling pathway inspired researchers to employ this amino acid as a free metal

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contrast agent (CA) in magnetic resonance imaging (MRI) for the detection of malignant glioma [12].

Metal-based complexes and nanoparticles, mostly based on gadolinium [13, 14] and iron [15-18], have been extensively studied as MRI contrast agents. However, emerging evidences revealed their overlooked side effects, resulting in the development of free-metal CAs. Compared metal-based CAs , 5-ALA has no toxicity at the standard dosage [19] and possesses high penetration across Blood brain barrier (BBB) through slight perturbation, making this free-metal CA as an intriguing option to enhance safety and improve the precision in diagnosis by MRI [12]. MRI of pathological tissues after 5-ALA administration could be considered as a complementary method with fluorescence imaging bearing the limitation of using during surgery and this arena seems to be worthwhile further investigations. The review provides an overview of the documents regarding the effect of 5-ALA on cellular iron metabolism and application of 5-ALA in MRI. Therefore, a comprehensive search for the terms of 5-ALA, PpIX, and iron metabolism was conducted using the PubMed, Scopus and Web of Science electronic databases. This work aims to review all documents related to the effect of 5-ALA on intracellular iron metabolism, with the approach of whether 5-ALA can be used as an MRI agent to detect the malignant regions. Firstly, we present a brief explanation regarding the iron availability effect on the Heme signaling pathway. Then, we focus on the impact of 5-ALA on the intracellular iron metabolism as well as the utility of 5-ALA in MRI. Finally, the possible application of 5-ALA in MRI and a prospect in this respect are discussed in this work.

The effect of Iron on the Heme signaling pathway

Heme biosynthesis pathway consists of eight steps occurring in the mitochondria and cytosol (Fig. 1). Initially, 5-ALA as a sole source in Heme biosynthesis [9, 20] is naturally synthesized in mitochondria via the condensation of glycine and succinyl–CoA [21] by the 5-ALA synthase (5-ALAS) [20]. After 5-ALA formation, porphobilinogen, hydroxymethylbilane, uroporphyrinogen III, and coproporphyrinogen III are generated in the cytosol sequentially. Coproporphyrinogen III is next imported into the mitochondria and is metabolized to PpIX, which is a fluorescent heterocyclic organic compound [22]. PpIX may be secreted outside of mitochondria through ATP-binding cassette transporters, such as ABCG2 [9]. However, PpIX is mainly converted to Heme through the enzyme ferrochelatase (FECH) catalyzing the entrance of ferrous iron into PpIX [23]. Mitoferrin imports iron into mitochondria [24]. And frataxin mediates iron delivery to FECH [25]. Synthesized Heme is then transported to the cytosol for incorporation into hemoglobin and other hemoproteins [26]. Heme oxygenase-1 (HO-1) which is induced by the elevated Heme level, catalyzes the degradation of remaining mitochondrial Heme producing equimolar quantities of biliverdin, free ferrous iron (Fe^{2+}), and carbon monoxide that would be reused [12, 27] (Fig. 1).

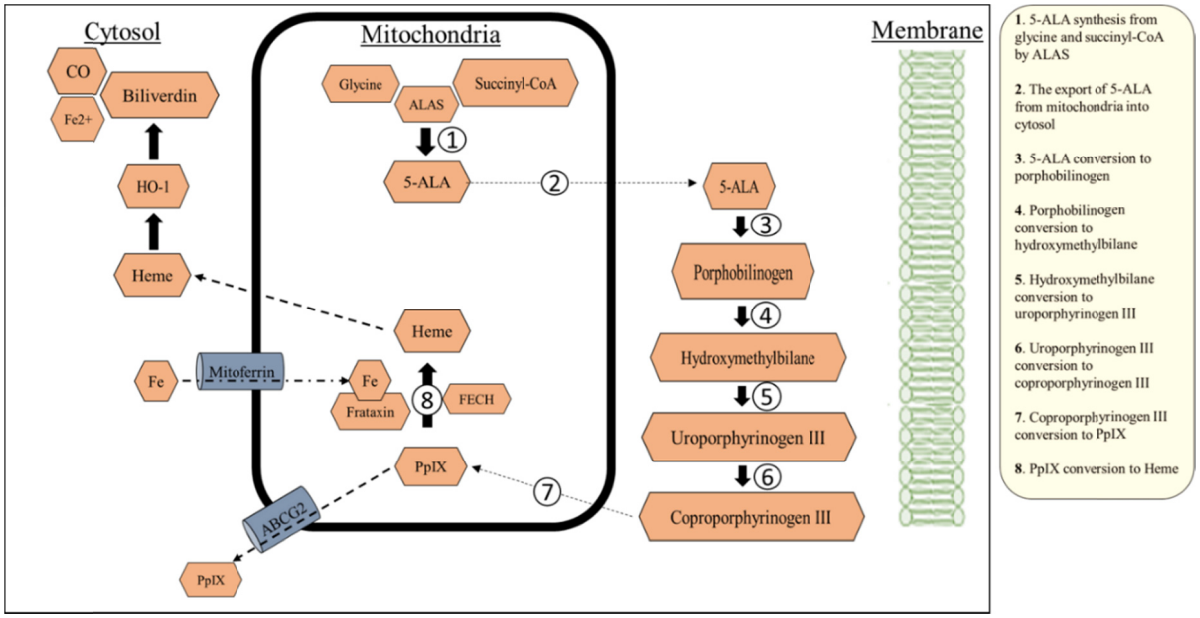

Figure 1. Schematic demonstration of the cellular Heme signaling pathway in eight steps.

Many previous studies have found that 5-ALA can be taken up by active transporters in the cancer cell membrane, such as PEPT1, PEPT2, GAT2, TAUT, and PAT1 [28-30], and administration of exogenous 5-ALA leads to the accumulation of PpIX in tumor cells [31, 32]. The rate of 5-ALA-mediated PpIX accumulation depends on various factors such as 5-ALA uptake by cells [29, 33], the ratio of 5-ALA conversion to PpIX, the export of PpIX [34, 35], Mitoferrin and frataxin activity, FECH activity [32], intracellular iron content [36] and amount of transferrin receptor (TfR) [37] (Fig. 2). Hagiya et al. demonstrated that 5-ALA induced PpIX accumulation depends on the balance between 5-ALA uptake and the excretion of PpIX in some types of gastric cancer cell lines [34]. Some studies revealed the effect of overexpression of mitoferrin [38] and frataxin, on decreasing the intracellular accumulation of PpIX. This effect is referred to the accessibility of iron to the Heme signaling pathway. It has been shown that the messenger ribonucleic acid (RNA) expression level of mitoferrin is lower in tumor cells than in normal cells [39], leading to a decrease in the mitochondrial iron level as well as PpIX accumulation in cancer cells. The accumulation of PpIX in tumor cells is also associated with the FECH activity [31], which is responsible for the incorporation of iron into PpIX to synthesis Heme in the situation of iron availability. Several studies suggested that FECH activity is diminished in tumor cells in comparison with normal cells leading to the accumulation of PpIX [40].

Moreover, it has been generally reported that the amount of intracellular labile iron ion is a substantial factor influencing tumor-selective PpIX accumulation [39]. Also, it has been shown that cellular and mitochondrial iron starvation leads to an increase in the expression of TfR on the tumor cell surface [36]. Therefore, TfR expression related to the iron deficiency in cancerous cells is considered as a marker for the intracellular level of PpIX.

Many studies have focused on the effect of iron availability or iron deficiency on the PpIX accumulation after exogenous administration of 5-ALA (Table 1). The studies concluded that the scarcity of iron leads to an elevated level of PpIX in cells which can be very useful in PDD and PDT [38].

Iron regulation in the Heme signaling pathway

Iron as *an important* nutrient of living cells is a vital cofactor for some essential functions, including oxygen transport, energy metabolism, DNA synthesis, electron transport, and Heme synthesis [22]. One of the critical functions of iron is associated with the Heme synthesis in cells. Heme is a coordination complex that contains an iron ion coordinated to PpIX, which is intermediated by FECH. Heme is an essential component for several types of hemoproteins, such as hemoglobin and myoglobin [41]. All cell types require iron for Heme synthesis. However, iron can be toxic in excess, and the iron level is controlled by some proteins like TfR and ferritin [42].

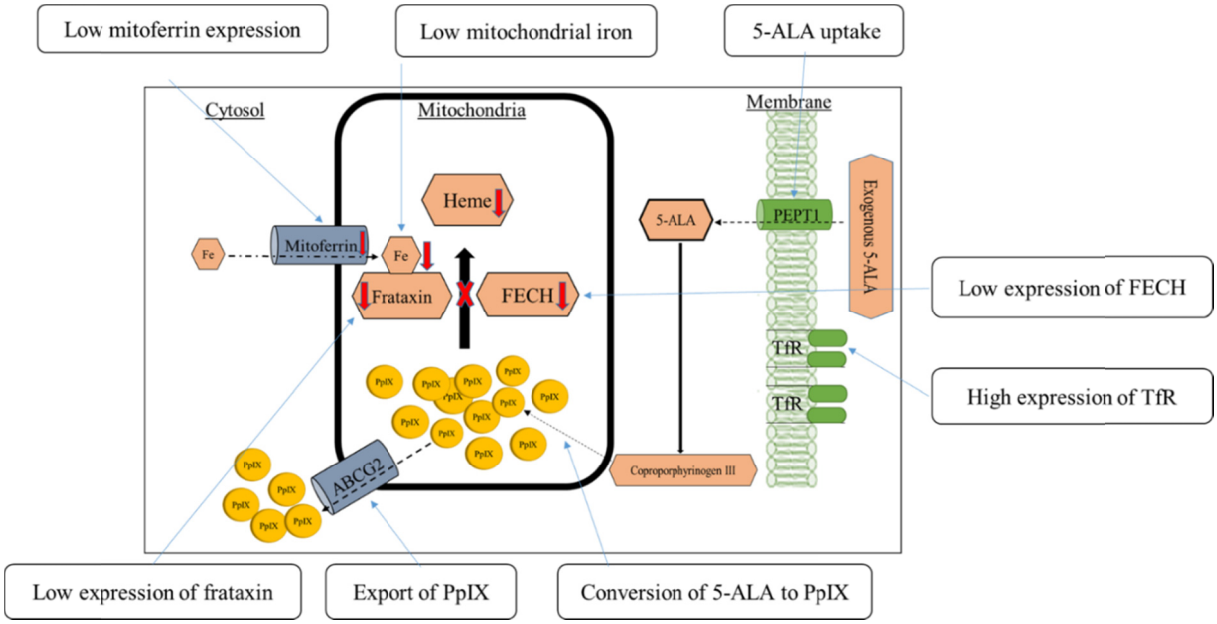

Figure 2. Schematic demonstration of the PpIX accumulation in tumor cells.

Table. 1. The previous studies performed regarding the effect of various factors, affecting intracellular iron content, on the PpIX accumulation.

Cell line	Treatment	Factors affecting intracellular iron content	Effect on PpIX accumulation	Ref
WiDr and V79	5-ALA+ EDTA or DFO		Iron chelator increased the cellular PpIX accumulation	[40]
PAM 212	5-ALA+ DFO	-	Iron chelator increased the cellular PpIX accumulation and photosensitizing process	[79]
CTCL	5-ALA	Expression of the TfR	Higher expression of TfR increased the cellular PpIX accumulation and photosensitizing process	[80]
EL-4	5-ALA+ DFO	-	Iron chelator increased the cellular PpIX accumulation and PDT efficiency	[81]
MCF7, MCF10A and MKN45	5-ALA+ sodium ferrous citrate	-	Iron source decreased the cellular PpIX accumulation	[39]
HeLa	5-ALA	Expression of HO-1 and Expression of mitoferrin	Higher expression of HO-1 and mitoferrin decreased the cellular PpIX accumulation	[38]

WiDr: Human colon adenocarcinoma; **V79**: Chinese hamster lung fibroblasts; **PAM 212**: Squamous *cell* carcinoma; **CTCL**: Human peripheral blood lymphocytes (PBL); **EL-4**: Murine thymic lymphoma; **MCF7**: Human breast adenocarcinoma; **MCF10A**: Normal human mammary epithelia; **MKN45**: Human gastric cancer; **HeLa**: Cervical cancer; **A431**: Epidermoid carcinoma.

In erythroid and non-erythroid cells, Heme metabolism and iron uptake are tightly regulated by TfR, while this regulation is different [43]. It has been shown that in erythroid cells, which are mainly responsible for Heme formation, intracellular Heme is the main factor controlling the number of TfR on the cell surface (Fig. 3). Previous studies indicated that TfR expression and intracellular iron uptake is increased by

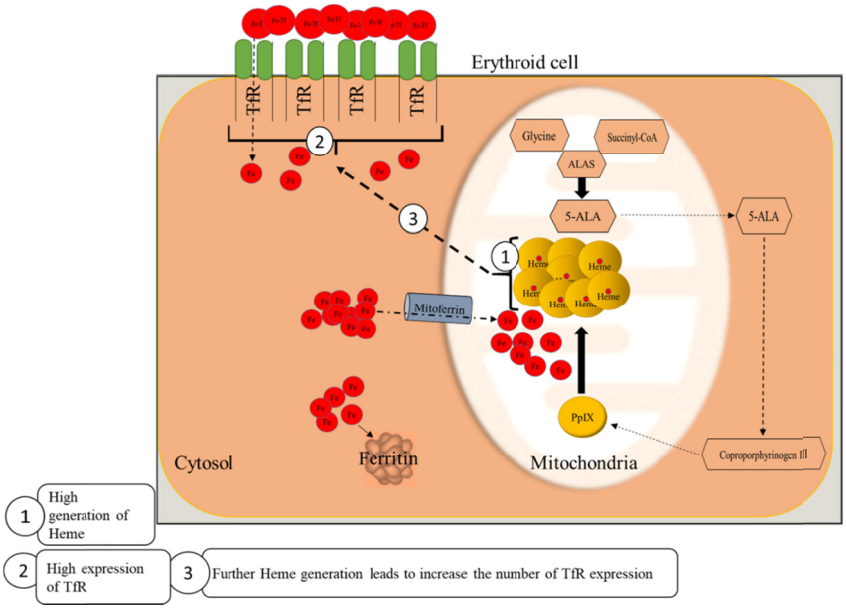

Figure 3. Schematic demonstration of the iron regulation in erythroid cells. The increase of the intracellular Heme content leads to enhance expression of the number of TfR and iron uptake.

further Heme synthesis to assure sufficient availability of iron for Heme generation in erythroid cells [10, 44].

However, the level of TfR expression is not affected by the intracellular Heme level in non-erythroid cells. Mitochondrial iron level is involved in the TfR expression on the cell surface and iron uptake, through activation of iron regulatory protein (IRP)/iron responsive element (IRE) system. IRPs and IREs are the proteins controlling iron homeostasis in the cell [43]. When a high amount of iron is available, the expression of TfR is prevented through the IRP/IRE system. On the other hand, in iron starvation, IRPs bind to IREs of TfR messenger RNA and increases the TfR synthesis. For instance, non-erythroid malignant cells possess a low intracellular iron level which is related to the competition between Heme synthesis and cellular growth process for iron consumption [31]. Hence, non-erythroid malignant cells having intracellular iron starvation are easily subjected to IRP/IRE system activation, leading to the high expression of TfR.

The effect of exogenous 5-ALA on cellular iron metabolism

To identify the impact of 5-ALA on Heme formation and iron metabolism, a series of studies were performed on erythroid and non-erythroid cells (Table 2).

The addition of exogenous 5-ALA bypasses the synthesis of endogenous 5-ALA as a first and rate-limiting phase in the Heme biosynthesis [45]. Heme synthesis in many cell lines, including human epithelial colorectal adenocarcinoma cells (Caco-2) [46], friend erythroleukemia cells [47], mouse macrophages (RAW264) [21], human gastric cancer cells (MKN28) [45], murine erythroleukemia cells (MEL) [48], human Caucasian chronic myelogenous leukemia (K562) [23], human primary glioblastoma (U-87) [12] and cervical cancer cells (Hela) [11] increased after exposure to 5-ALA (Fig. 4). Notably, the combination of 5-ALA and sodium ferrous citrate promoted Heme synthesis due to the availability of the required iron [45, 46].

5-ALA can also affect iron metabolism through its effect on the level of TfR expression in erythroid cells (Fig. 4). It has been confirmed that TfR expression and iron uptake are stimulated by 5-ALA administration [10] to provide sufficient iron for Heme synthesis [48, 49]. Treatment of MEL cells with 5-ALA increased TfR expression and also iron incorporation into the cells [10] Furthermore, the incubation of murine erythroleukemia cells with 5-ALA after 48 h led to a dose-dependent increase in TfR messenger RNA levels.

The up-regulation of TfR synthesis was observed in non-erythroid cells treated with 5-ALA. Activation of the IRE/IRP system in the situation of iron starvation was suggested as a possible mechanism of the up-regulation. It has been shown that the incubation of cervical cancer *cells* (Hela cells) with low concentration of 5-ALA leads to an increase in the number of TfR [11]. Besides, Cho et al. evaluated the impact of 5-ALA on human primary glioblastoma (U-87) cell line as well as an animal model [12]. The results showed that 5-ALA administration increased the intracellular iron concentration in both U-87 cells and animal xenograft model through promoting the synthesis of Heme.

Moreover, it has been demonstrated that 5-ALA not only could affect TfR expression, but also ferritin content (Fig. 4). 5-ALA can release iron from ferritin [50, 51] as well as mobilize ferritin-iron through inducing oxidative stress, confirmed by *in vitro* and *in vivo* analyses, respectively [52, 53]. On the other hand, 5-ALA can stimulate the further synthesis of ferritin by activating the IRP system. It has been suggested that 5-ALA may increase the non-Heme iron content in rat liver leading to the formation of numerous ferritin granules [54] observed in liver biopsy samples of patients suffered from acute intermittent (AIP).

The effect of intracellular iron-containing molecules in MRI

Mainly, two forms of the iron complex are known to appear in the cells, including Heme and non-Heme molecules [55]. As mentioned in previous sections, while Heme contributes to hemoglobin formation, iron-containing non-Heme complexes like ferritin and hemosiderin are responsible for intracellular iron storage [56]. It has been suggested that ferritin and hemosiderin are only types of intracellular iron complexes which are detectable in MRI [57]. Many studies have documented the MRI capability for detecting iron deposition in the body [58-61].

Intracellular iron overload is indirectly identified by the susceptibility effects of the iron on the shortening of water proton MR relaxation times. Proton nuclei are the main constituent of tissues, producing a magnetic field with the ability to interact with an external magnetic field (B_0) (Fig 5. a). In the presence of an external magnetic field, proton nuclei align in a direction parallel to the magnetic field (Fig 5. b). After the emission of radiofrequency (RF) pulses, the proton nuclei absorb the RF energy (Fig 5. c). Turning off the RF pulse, the absorbed energy by nuclei is released and return to the normal state by two characteristic parameters called T1 and T2 relaxation times in the longitudinal and transverse planes, respectively (Fig 5. d). In the case of accumulated iron acting as a magnet, the magnetic field inhomogeneity is generated in the surrounding tissues. Therefore, the moving water protons experiencing

Cell line or sample	Treatment	TfR	Ferritin	HO-1	Heme	Iron uptake/ accumulation	*In vitro*/ *In vivo*	Ref
MEL	5-ALA	Increased	-	-	Increased	Increased	*In vitro*	[10]
Liver biopsy samples obtained from AIP patients	5-ALA	-	Increased	-	-	Increased	*In vivo*	[54]
Caco-2	5-ALA+ sodium ferrous citrate	-	-	Increased	Increased	-	*In vitro*	[46]
MELC	5-ALA	-	-	-	-	Increased	*In vitro*	[82]
MELC	5-ALA+Hemin	-	-	-	Increased	Decreased	*In vitro*	[82]
MEL	5-ALA	Increased	-	-	-	Increased	*In vitro*	[48]
K562	5-ALA	-	-	-	-	Increased (in a dose dependent manner)	*In vitro*	[23]
Horse spleen ferritin	5-ALA	-	Increased	-	-	-	*In vitro*	[50]
Horse spleen and rat liver	5-ALA	-	Increased	-	-	Iron accumulation in the liver of 5-ALA-treated rats was observed	*In vitro*/ *In vivo*	[51]
Brain tissue	5-ALA	-	-	-	-	total non-Heme iron in the cortex was increased	*In vivo*	[83]
Brain tissue	5-ALA	-	Ferritin in the cortex and in striatum increased	-	-	Total iron in the cortex was increased	*In vivo*	[83]
U-87	5-ALA	-	-	-	Increased	Increased	*In vitro*/ *In vivo*	[12]
MEL	5-ALA	Increased	-	-	-	Increased	*In vitro*	[10]
RAW264	5-ALA+ sodium ferrous citrate	-	-	Increased	Increased	-	*In vitro*	[19]
MKN28	5-ALA+ sodium ferrous citrate	-	-	-	Increased	-	*In vitro*	[84]
Hela	5-ALA	Increased	-	-	Increased	-	*In vitro*	[11]

Caco-2: Human epithelial colorectal adenocarcinoma; **MELC**: Mouse erythroleukaemia; **K562**: Human Caucasian chronic myelogenous leukemia; **U-87**: Human primary glioblastoma; **RAW264**: Mouse macrophage; **MKN28**: Human gastric cancer; **HeLa**: Cervical cancer.

different magnetic field become desynchronized from each other (Fig 5. e) [62]. This phenomenon leads to the significant alterations in tissue relaxation times. While T_1 decreases only moderately, T_2 or T_2* demonstrates a substantial decrease [63].

ALA in MRI

Despite several studies performed on the potential of 5-ALA for altering cellular iron metabolism, only one research studied the potential application of 5-ALA in MRI through the susceptibility effect of iron. Cho et al. hypothesized that 5-ALA may be useful for malignant glioma cell detection by MRI via promoting the synthesis of Heme [12]. For the *in vitro* study, U-87 cell was treated with 5-ALA for 6 h and intracellular concentrations of iron and Heme were then measured.

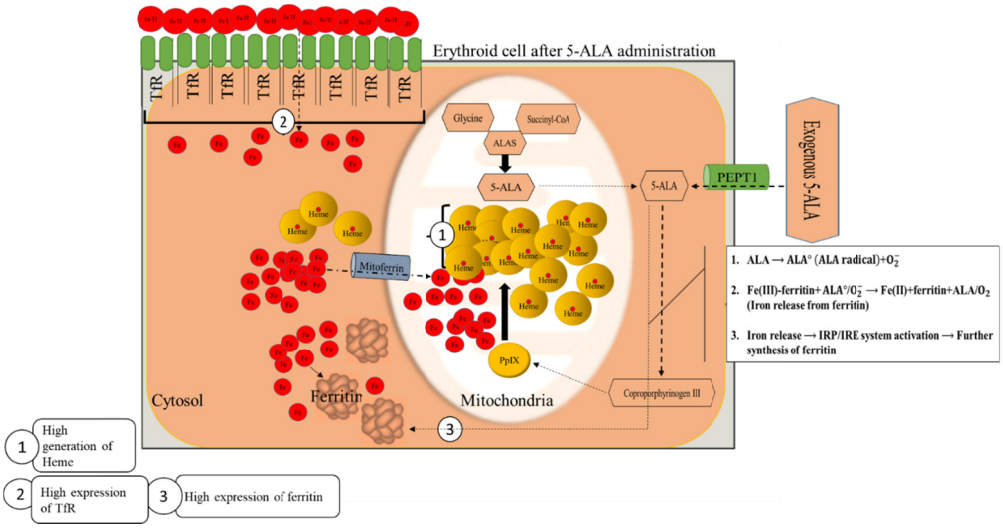

Figure 4. Schematic demonstration of the effect of exogenous 5-ALA on erythroid cells. 5-ALA can affect intracellular iron metabolism, and increase the level of Heme, TfR, and ferritin expression in erythroid cells.

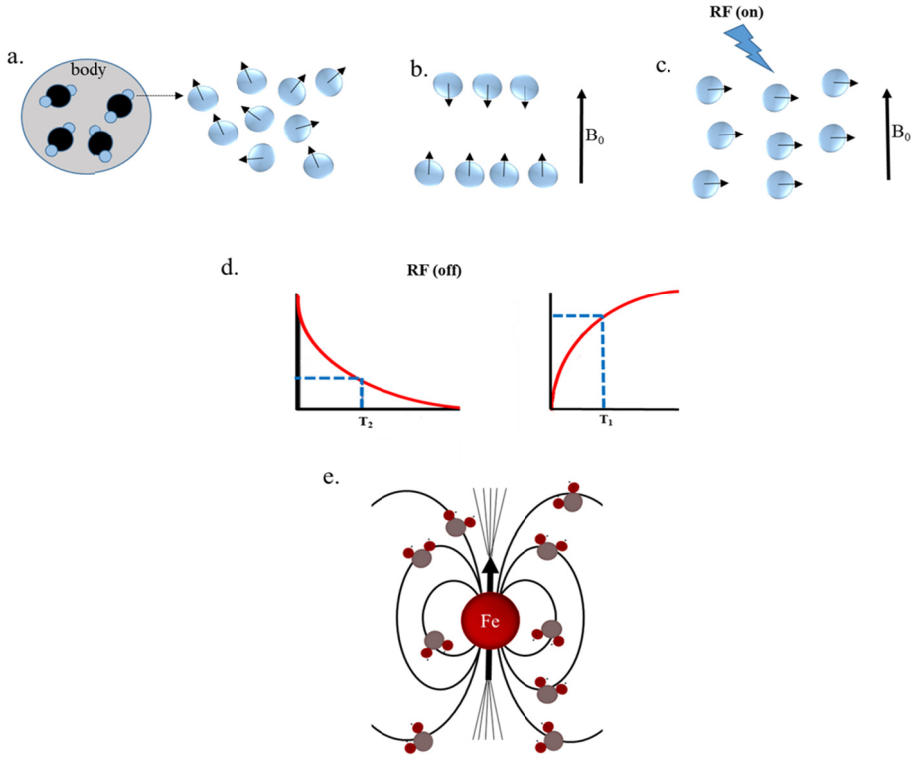

Figure 5. Proton nuclei are the main constituent of tissues, producing a magnetic field with arbitrary direction (a). In the presence of an external magnetic field, proton nuclei align in a direction parallel to the magnetic field (b). After the emission of radiofrequency (RF) pulses, the proton nuclei absorb the RF energy (c). Turning off the RF pulse, the absorbed energy by nuclei is released and return to the normal state by two characteristic parameters called T1 and T2 relaxation times (d). Magnetization of the iron interacting with nearby spins in water causes the shortening of the transverse relaxation time (e).

Furthermore, for *in vivo* study, T_2*-based protocol was used to create MR images, before and 24 h after 5-ALA administration and the T_2* value was mapped. Also, the accumulated iron in tumor tissue was evaluated using

Prussian blue staining. The results showed that the intracellular level of Heme and total iron increased 24 h after 5-ALA treatment. Moreover, mouse brain imaging demonstrated a lower median T_2* value (14.2 ms) compared to that in the control group (23 ms) 24 h after 5-ALA administration, which was confirmed by iron measurement results. (Fig. 6. a, b, c).

In this study, the authors suggested that the leading cause of the decrease in T_2* value could be the susceptibility effect of the Heme accumulation in glioma after 5-ALA administration. Heme is an iron complex showing T_1-sensitive effect on MRI at low concentrations, due to its paramagnetic property. Mainly, metal-complexes such as iron and gadolinium complexes represents enhanced signal intensity on T1-based MR images [64]. However, the accumulation of the iron complexes with high concentrations have been demonstrated to create signal loss on T_2*-based MR images, due to inducing further rate of proton spin dephasing [65]. Nevertheless, Heme was suggested to be catalyzed to carbon monoxide, biliverdin and free iron through the expression of Heme oxygenase enzyme. Even low elevation in intracellular Heme content may induce HO which is able to lower the intracellular Heme content [11]. Notably, the promoted ferritin accumulation in the cells treated with 5-ALA [50] as well as further synthesis of ferritin induced by the iron released form the Heme was reported by some

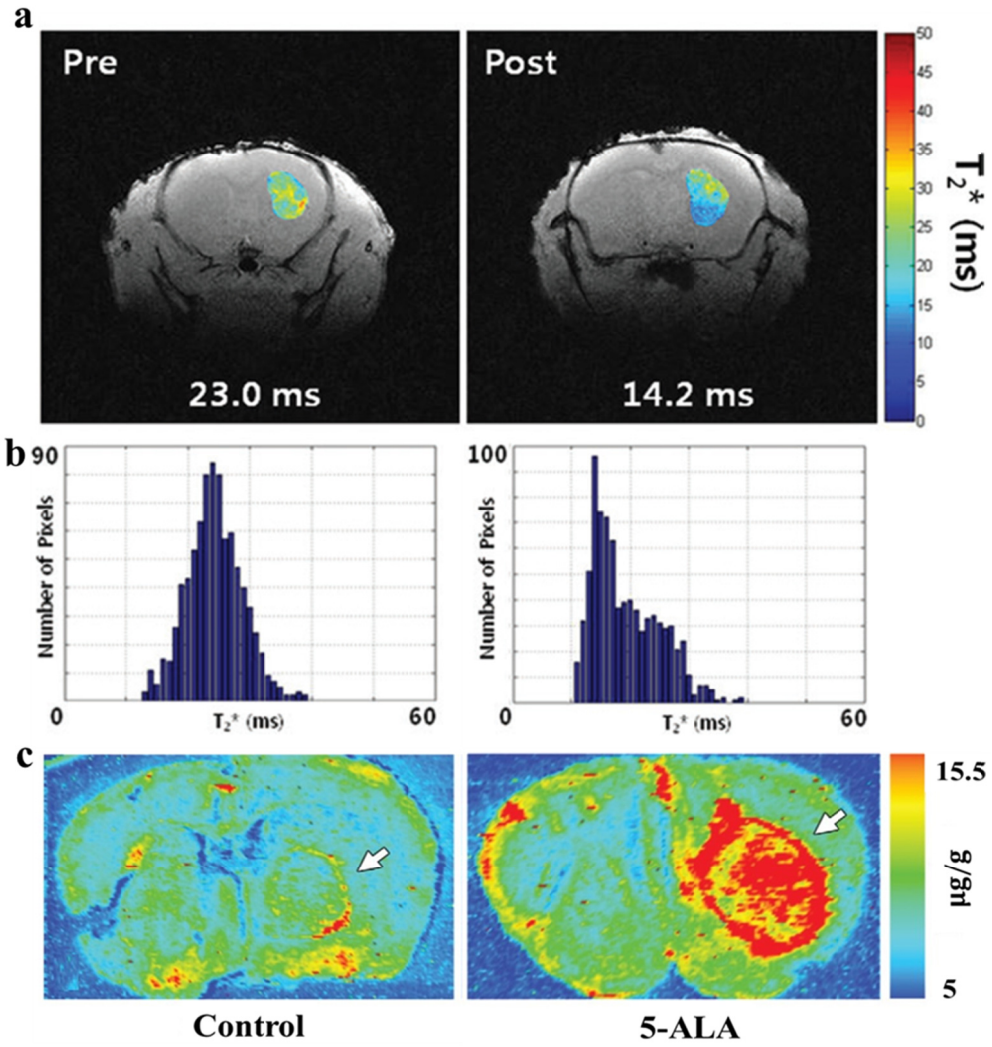

Figure 6. (a) The T_2* mapping of MR images obtained from the previous study regarding the application of 5-ALA for MRI based on the iron accumulation induced 5-ALA [12]. **(b)** The histograms of T_2* mapping belong to the region of interest of the above images, representing a decrease in the median T_2* value of brain tumor after administration of 5-ALA (23 versus 14.2 ms). **(c)** Prussian blue staining of the sectional tumor tissue, before and 24 h after 5-ALA administration. With permission from the Radiological Society of North America (RSNA®). (Cho H R, Kim D H, Kim D, et al. Radiology 2014;272:720-730).

previous studies [66]. Therefore, it is possible that not only Heme accumulation is contributed to creating MR images contrast after 5-ALA application, but also ferritin may be thought to be the source of susceptibility resulting in a decrease in T_2* value. It is conceivable that the signal intensity on T_2-based MR images could be decreased by increased level of intracellular ferritin containing 4,500 iron ions with superparamagnetic property. The MRI signal loss originated form intracellular ferritin accumulation has been shown by many previous studies [67, 68]. However, the effect of 5-ALA on the expression of proteins involved in cellular iron metabolisms like ferritin and TfR has not been identified so far in glioma cells, and further efforts should be taken to assess this issue.

One of the main limitations of the study performed by Cho et al. was the application of a high dose of 5-ALA (100 mg/kg) than the standard one (20 mg/kg) which could be resulted in toxic side effects. No sufficient MR images contrast was achieved using the standard dose of 5-ALA. The authors performed MRI of mouse brain tumor 24 h after 5-ALA administration, based on the *in vitro* results, without *in vivo* optimization of the imaging time. *The* required *dose of* 5-ALA for creating MR images contrast and the time of MRI after 5-ALA administration are interdependent parameters, and desirable contrast on MR images with the standard dose of 5-ALA may be achieved using the optimized imaging time for 5-ALA–enhanced MRI.

Low intrinsic sensitivity in MRI with 5-ALA may also be attributed to the limited uptake of 5-ALA or limited uptake of iron into tumor cells. 5-ALA is a polar amino acid. Therefore, its absorption into the cells can be hampered by the hydrophilic nature of 5-ALA [69]. To circumvent this obstacle, many strategies, including derivatization, the use of different drug delivery systems, such as esterified 5-ALA prodrug derivatives, liposomes [70], and the methyl and hexyl ester derivatives may be useful to enhance the intracellular 5-ALA uptake [71]. Moreover, limited iron accumulation by tumor cells in 5-ALA-based MRI may be further modified using iron chelators before 5-ALA administration due to the ability of iron chelators to decrease the intracellular labile iron [40]. It may be useful for inducing further IRP/IRE system activation, arising from intracellular iron starvation, leading to TfR expression and iron accumulation enhancement. Many previous studies confirmed the effect of iron chelators like deferoxamine mesylate (DFO) on the increase of PpIX accumulation due to a decrease in the intracellular labile iron (Table 1). We hope that further investigation regarding the application of 5-ALA in MRI in accompanied with an iron chelator will facilitate the application of 5-ALA in MRI with the standard dose. This strategy may enhance the intrinsic sensitivity of 5-ALA-based MRI. Moreover, not just intrinsic sensitivity enhancement may be effective to the feasible application of 5-ALA as a CA in MRI, but also the MRI detection sensitivity improvement can be useful in this respect.

MRI sensitivity is also associated with the performed imaging protocol. To detect accumulated iron in tissues, there are three main methods, including T_2* as a qualitative method demonstrating susceptibility effect of the iron [72], susceptibility-weighted imaging as a qualitative method accompanied with additional image processing to enhance image contrast [73], and quantitative susceptibility method (QSM) that directly quantify susceptibility values of the iron [74]. All of these protocols demonstrate susceptibility effects. However, the sensitivity of QSM as a quantitative tool for directly measuring the susceptibility value of the tissue is higher than that of two other protocols [75]. Application of quantitative assessment of the iron accumulation using QSM may be a promising strategy providing a more sensitive and accurate estimation of iron changes creating sufficient contrast in MRI. The ability of QSM regarding the quantifying iron deposition, confirmed by previous studies, suggests the possible efficacy of this method as an alternative for the T_2* method.

Another study regarding 5-ALA utility in MRI was performed by Yamamoto et al. in 2017 [76]. In this study, it was proposed the potential of 5-ALA-induced PpIX for enhancing the T_2 signal intensity in high-grade glioma. So, patients bearing Glioblastoma multiforme (GBM) and anaplastic oligodendroglioma (AO) were imaged with an MRI scanner using T_2 weighted imaging protocol, at before and 2.5 h after 5-ALA administration (20 mg/kg). A modified operating microscope fluorescence was then used to analyze the quantities of PpIX in tumors. All GBM tumors with strong fluorescence exhibited the augmented MRI T_2 signal after 5-ALA administration, while no T_2 signal enhancement was observed in the AO group, *which has no fluorescence. It was thereby indicated* that there is a relationship between the accumulation of PpIX in tumor cells and T_2 signal enhancement. The authors suggested that the T_2 signal enhancement after 5-ALA administration is possibly due to water solubility of 5-ALA contrary to the water insolubility of PpIX. This difference may be responsible for increasing the water content in tumor cells following the conversion of 5-ALA to PpIX and changing the T_2 signal on MR images. However, the authors of this study believed that the underlying mechanism associated with T_2 signal

enhancement after 5-ALA administration was not fully understood. As well as the effect of 5-ALA on changing the T_2 signal intensity for clinical application is not sufficient. Therefore, especially the ability of 5-ALA to changing the T_2 effect in the infiltrative area of tumors with weak PpIX accumulation will be questioned. The previous studies demonstrated that the amount of PpIX accumulation could be an indication of tumor malignancy *in a different area of the tumor*. Active tumor cells show strong fluorescence, but the infiltrative area in tumor margin possess weak fluorescence intensity [77]. This PpIX accumulation diversity in the tumor may lead to induce different water content in various areas of tumors, providing unique capabilities for discriminative detection of tumor cells using T_2 weighted MR images contrast. Accordingly, Further studies regarding the utility of 5-ALA in MRI using water-sensitive sequence like diffusion-weighted imaging as an MRI technique, based on measuring the random motion of water molecules [78], will become necessary.

Conclusions and prospects

In this review, we attempt to collect available documents as for the effect of exogenous 5-ALA on Heme biosynthesis and iron metabolism, justifying the utilization of 5-ALA in MRI. Foremost, to achieve the overall conclusions, it seems necessary to provide the highlights from the literature, including the difference between erythroid and non-erythroid cells for iron regulation, the effect of 5-ALA on the Heme generation and the expression of iron metabolism-related genes. Erythroid cells are mainly responsible for Heme formation to synthesis the hemoglobin. Addition to erythroid cells, all aerobic cells are also able to produce Heme through iron uptake. However, there is a characteristic difference between erythroid and non-erythroid cells regarding their cellular iron regulation for Heme formation. The amount of iron uptake is regulated by Heme level in erythroid cells and by IRE/IRP system activation resulting from iron deficiency in non-erythroid cells. Despite this difference, the addition of exogenous 5-ALA to the cells leads to stimulate further Heme biosynthesis, in circumstances where the iron ion is accessible in erythroid and non-erythroid cells. Moreover, it has been shown that the TfR expression as a cellular iron uptake mediator and the ferritin expression as a cellular iron storage protein could be increased after 5-ALA application in erythroid cells and cancerous non-erythroid cells.

Considering the mentioned points, it can be probably concluded that there are three essential factors, determining whether 5-ALA would affect the iron metabolism as well as iron uptake in each cell types. The first one is the variable affinity of 5-ALA toward different cell lines. Many cell lines, including hepatocytes, macrophages and especially tumor cells like HGG have a high affinity towards 5-ALA. It is known that 5-ALA can absorb by cancer cells and convert into fluorescent PpIX, leading to the application of 5-ALA as a fluorescent CA for intrasurgical malignant glioma detection. The second one is the inherent involvement of the cells in metabolism and homeostasis of iron through absorption and storage. Mainly, erythroid cells, liver and tumor cells possess high iron metabolism by regulating the TfR and ferritin expression. Further explanations about iron metabolism in glioma cells, it is essential to note that U-87 cells are originated from astrocytes, which play an important role in the uptake, storage and release of iron in the brain. The third factor is dedicated to the necessity of IRP/IRE activation to enhance TfR expression in non-erythroid cells, in the situation of iron starvation, which is mainly occurred in tumor cells with lower iron level in mitochondria compared with those in normal cells, because of the high iron consumption for cell proliferation.

Therefore, cancer cells like glioma possessing high affinity toward 5-ALA, having inherent involvement in iron metabolism and iron starvation, leading to IRP/IRE system activation, may be considered as the main target for the accurate diagnosis using MRI with 5-ALA via susceptibility effect of accumulated iron. Generally, the specific impact of 5-ALA on iron metabolism of cancer cells, leading to the higher accumulation of iron *in these cells* compared with normal cells, make it possible to produce a *discriminative diagnosis* of tumor region and enhances the detection specificity. Moreover, the higher sensitivity of 5-ALA to be entered into the malignant area of tumor with negligible damage in BBB compared with the Gd-based CAs in MRI, confirmed in 5-ALA-based fluorescence imaging, *suggest 5-ALA as a valuable candidate in cancer diagnosis.* Also, the therapeutic application of 5-ALA in PDT together with diagnostic properties of 5-ALA as a dual CA in fluorescence and MRI, make this valuable amino acid as a promising agent in cancer theranostic and emerge the need for further investigations on this subject.

Acknowledgements

Authors would like to acknowledge Tehran University of Medical Sciences (TUMS), Tehran, Iran, for providing the best services for study and analysis

regarding this review article.

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