Hemoglobin Conjugates with Antioxidant Enzymes (Hemoglobin-Superoxide Dismutase-Catalase) via Poly(Ethylene Glycol) Crosslinker for Protection of Pancreatic Beta RINm5F Cells in Hypoxia

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A low p50 hemoglobin (Hb) (p50 indicates O₂ tension at which Hb is half-saturated)-based oxygen carrier conjugated to antioxidant enzymes via dicarboxymethylated poly(ethylene glycol) (PEG) linker may have the beneficial effect in protecting pancreatic beta cells from severe hypoxia at transplantation sites. In this study, the oxygen dissociation curves, Hill plots, Bohr Effect, and oxygen content of Hb conjugates were measured. The protective effect due to incubation of Hb-conjugates (Hb/PEG molar ratio 1:10) with pancreatic beta cells (RINm5F) against hypoxia (6%, 3%, and 1% oxygen) was evaluated by an MTT assay and confocal microscopy. Quantitatively, Hb conjugates with antioxidant enzymes offered statistically significant protection ($p < 0.01$, increased viability ~80%) from hypoxia compared to control cells in 1% oxygen environment. Confocal images also showed that the low p50 Hb conjugates with antioxidants protected RINm5F cells from hypoxia.

Introduction

PANCREATIC BETA CELLS under diabetic conditions are susceptible to reactive oxygen species (ROS) such as superoxides, which leads to the islets dysfunction.¹ ROS are known to be generated by factors such as high/low glucose levels and/or hypoxia.^{2,3} Low oxygen tension (between 40 and 5 mm Hg, which corresponds to 6% to 0.5% oxygen partial pressures) has been experimentally shown to exist in transplanted rat pancreatic islets irrespective of the transplantation site (liver, kidney, and spleen).⁴ High sensitivity of islets to these hypoxic stresses during islet isolation, culturing/maintenance, and post-transplantation is one of the major obstacles to successful islet transplantation.⁵ Low islet revascularization post-transplantation is also shown to cause beta cell death *in vivo*.⁶ Human islets and a beta cell-line (MIN6) exposed to hypoxia (1% oxygen) showed loss of viability due to necrosis within 24 h.⁷ Under these low pO₂ conditions, hypoxic cell mitochondria also produce ROS primarily due to altered cellular redox potential caused by the slowing of respiratory chain reactions such as decreased capacity of cytochrome C to trap oxygen.⁸ *In vivo*, tissues and cells with intact blood vessels sense change in pO₂ through different mechanisms and adapt to altered oxygen level by changing adenosine triphosphate (ATP) production, expressing hypoxia-induced factor, increasing blood flow, and forming new blood vessels.⁹ In hypoxic stress, intracellular

pH may be decreased by acidic environment,¹⁰ and increased demand for oxygen. Therefore, the design of an appropriate oxygen carrier that can release higher amount of oxygen under low partial oxygen has practical applications in islet cell transplantation.

The design goal in most hemoglobin (Hb)-based artificial oxygen carriers is to synthesize Hb with similar oxygen-carrying properties of Hb (inside red blood cells [RBCs]) with a typical sigmoidal cooperative oxygen binding. In perfluorocarbon-based (PFC) oxygen delivery systems, release of oxygen is linear in contrast to the more desirable sigmoidal curve with natural Hb. Low stability, toxicity of the PFC emulsions, and the loose binding of oxygen by van der Waals interactions compared to tight chemical bonding in natural Hb are other significant shortcomings with PFCs.¹¹ These issues cause instantaneous release of oxygen by PFCs and may not provide long-term benefit in hypoxia when compared to low p50 (high affinity) oxygen systems.¹²

To overcome the related problems of hypoxia and hypoxia-induced free radical stress and/or environmental radicals, a combined protective effect in a suitable oxygen-carrier is highly desirable. There exists an unmet clinical and technological need for a multifunctional oxygen-carrier with low p50 capable of releasing oxygen and an inbuilt antioxidant defense mechanism that protects Hb from hypoxia-induced ROS and cells from hypoxia. Polymerization and cross-linking offer a practical way to alter p50 that depends on the

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crosslinking agent and may be mediated by chemistry of the conjugation.¹³ A typical approach described in the literature (glutaraldehyde as a conjugating agent) resulted in high p50, which is more relevant in normal conditions than the low p50 conditions encountered at the sites of transplantation.¹⁴ Also, glutaraldehyde crosslinking is shown to promote unwanted effect of vasoconstriction¹⁵ and release of the polymeric linker by degradation exhibiting cytotoxicity.¹⁶

Our previous *in vitro* studies investigated the protective effect of crosslinked Hb-superoxide dismutase-catalase (Hb-SOD-CAT) at room temperature and demonstrated that antioxidant mechanism offered significant protection on Hb against free radical stresses.¹⁷ The antioxidant enzyme SOD catalyzes the reduction of superoxide radical to hydrogen peroxide and oxygen and CAT catalyzes the breakdown of hydrogen peroxide to water and oxygen. Results have shown that linking of these enzymes to Hb by poly(ethylene glycol) (PEG) only caused minimal loss of enzymatic activity post-conjugation (SOD activity >70% and CAT >90%).¹⁷ These enzymes may also provide protective effect from free radicals induced during hypoxia and therefore help protect cells or tissues.¹⁸ In this research approach, oxygen binding and release capability was investigated that helps in understanding the need for tailoring low p50 Hb conjugates with antioxidant enzymes, for specific tissues engineering applications such as protecting cells/tissues in hypoxia and/or hypoxia-induced free radical stress. The protective ability of these conjugates on the pancreatic beta cell line (RINm5F) under hypoxic conditions (6%, 3%, and 1% oxygen) was tested.

Experimental Methods

Materials

Bovine RBC suspension was purchased from Innovative Research (IC100-0410). PEG (2 kDa), Superoxide dismutase from bovine erythrocytes (SOD-S7571), bovine liver catalase (CAT-C40), cell culture media RPMI-1640, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), acridine orange (AO), propidium iodide (PI), potassium tertiary butoxide, ethyl bromoacetate, *N*-hydroxy-succinimide (NHS), and *N,N'*-dicyclohexylcarbodiimide (DCC) were purchased from Sigma Chemical Co. Penicillin-streptomycin, fetal bovine serum (FBS), and Trypsin-EDTA-0.25% solution were purchased from Gibco Co. Dialysis membranes were purchased from Spectrum Labs. Amicon™ Ultra-15 Centrifugal Filter Units, 50 and 100 kDa, were purchased from Millipore Corporation.

Conjugation of Hb with SOD and CAT

Hb was isolated from freshly collected bovine RBCs as described previously; its purity was verified by employing sodium dodecyl sulfate–polyacrylamide gel electrophoresis and quantified by the Drabkins method.¹⁹ PEG (2 kDa) was also chemically modified as previously described¹⁹; potassium tertiary butoxide and ethyl bromoacetate was used to modify the hydroxyl end groups on PEG to ethyl-protected carboxymethylated PEG. Basic hydrolysis of this intermediate PEG compound resulted in mixture of PEG-carboxylic acids. Further purification was done by ion-exchange chromatography. PEG-dicarboxylic acid used in conjugation was

activated by employing NHS and DCC. Bovine Hb was conjugated with and without two antioxidant enzymes (SOD and CAT) in conjugation ratios of Hb/PEG (1:5, 1:10, 1:15, 1:20, and 1:30) as described previously.¹⁷ The ratio between SOD and CAT was fixed at 30,000 to 300,000 enzyme units in conjugation reactions with antioxidant enzymes. The conjugation reaction was carried out by stirring for 3 h at 4°C and the reaction product was dialyzed for 24 h. By employing Amicon™ centrifugation filters, the desired concentration of the conjugates (1 mM) was achieved. A 0.22 µm size syringe was used to filter the product and stored at 4°C until further use.

Measurement of oxygen Hb equilibrium curves

The oxygen-affinity of Hb-based conjugates was determined experimentally by using a commercially available HEMOX Analyzer and HEMOX Analytical Software (TCS Scientific Corporation).²⁰ Oxygen equilibrium curve, in both oxygen association and dissociation phases, was measured by using dual wavelength absorbance spectroscopy. A Clark electrode was used to directly measure the partial oxygen pressure in the sample cuvette. Oxygen hemoglobin equilibrium curves (OEC) was obtained by plotting changes in optical absorbance on the X-axis by measuring maximum absorbance at 560 nm and a reference at 570 nm with the different partial pressures on y-axis. For all experiments, 0.25 mM equivalent Hb solution was placed in a cuvette containing the hemox solution and the temperature of the mixture equilibrated to 37°C by continuous heating and stirring. Simultaneously, the sample was completely oxygenated by using ultra-high-pure oxygen. A deoxygenation curve was obtained by replacing oxygen with ultra-high-pure nitrogen gas in conditions where Hb becomes deoxygenated ($\text{HbO}_2 \leftrightarrow \text{Hb} + \text{O}_2$). An oxygenation curve is obtained by replacing nitrogen with oxygen after the partial oxygen pressure had almost reached zero. All the samples are tested at three different pH values (6.5, 7.4, and 8.0) to determine the Bohr Effect.

The oxygen equilibrium curve was obtained by stepwise curve-fitting analysis from the Adair equation, which assumes sequential binding of oxygen to Hb molecule (four available binding sites, each with a different equilibrium constant).²¹ The y values are calculated from the estimated Adair constants measured at each partial pressure and accordingly by scaling the data points. Stepwise Adair equation is as follows:

$$y = \frac{a_1 p + 2a_1 a_2 p^2 + 3a_1 a_2 a_3 p^3 + 4a_1 a_2 a_3 a_4 p^4}{4(1 + a_1 p + a_1 a_2 p^2 + a_1 a_2 a_3 p^3 + a_1 a_2 a_3 a_4 p^4)}$$

where p is partial oxygen pressure, y represents the fractional oxygen saturation, and a_1 , a_2 , a_3 , and a_4 are binding constants.

Hill plots were obtained by plotting $\text{Log} \frac{y}{(1-y)}$ versus $\text{log}(p\text{O}_2)$ values. The p50 value (partial oxygen pressure at which Hb molecule is 50% saturated) was calculated by extrapolating the x -axis value (the partial pressure) to y -value (50% fractional saturation). Hill slope at p50 (n_{50}) is obtained by calculating the slope at p50.

Bohr coefficient was estimated using the Wyman equation.²² It is given by $\frac{\Delta \log p50}{\Delta \text{pH}}$, which estimates the average

number of Bohr protons released (expressed as negative value) per binding site upon full oxygenation.

Cell culture and hypoxia (6%, 3%, and 1% O₂) experiments

RIN5mF cells were used to estimate the effectiveness of Hb conjugates as oxygen carriers in hypoxic conditions (6%, 3%, and 1% oxygen). RINm5F cells (1×10^5 cells per well) were seeded by using RPMI media containing 10% FBS and were allowed to attach to plates by incubating overnight at 37°C with 5% CO₂ and 95% air in normoxic conditions (21% oxygen). At 24 h, the medium was changed with fresh RPMI media containing Hb formulations with and without antioxidants (optimized conjugating ratio 1:10 Hb/PEG). This ratio was chosen based on our previous results showing low methemoglobin formation (a poor oxygen carrier).¹⁷ The following groups were selected for the demonstration of the activity of Hb-conjugates: (1) cells with RPMI medium only, (2) cells with RPMI medium and 0.1 mM unconjugated Hb, (3) cells with RPMI medium and 0.1 mM conjugated Hb-Hb, and (4) cells with RPMI medium and 0.1 mM Hb conjugated with antioxidants (Hb-SOD-CAT). Hypoxia environment (6%, 3%, and 1% oxygen) was achieved by using a tri-gas incubator (ThermoFisher Scientific Inc.) with continuous injection of an appropriate amount of ultra-high-pure N₂ to reach the target oxygen levels; at equilibrium, incubator was saturated with the required % of O₂, (6% or 3% or 1%) and 5% CO₂, and balance with N₂. Cells were cultured for 48 h in triplicate for each group by placing into the hypoxic incubator.

Oxygen content

Oxygen content at any given partial oxygen pressure in the medium is calculated by using the following formula:

$$\text{Oxygen content O}_2 \frac{\text{mL}}{\text{L}} = \left[\left(\text{Total Hb in } \frac{\text{grams}}{\text{L}} \right) \times (1.34) \times (\% \text{HbO}_2 \text{ expressed as fraction}) \right] + [(p\text{O}_2 \text{ in mm Hg}) \times (0.03)]$$

where, 1.34 mL of oxygen is bound by a gram of Hb (Hufner's number), % HbO₂=amount of oxygen combined with Hb; pO₂=partial pressure of dissolved oxygen in mm Hg, and 0.03 represents conversion of 1 mm Hg of dissolved oxygen to mL/L.^{23,24}

MTT assay for cell viability

Cell viability was determined by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. After a defined treatment incubation period of 48 h, MTT was added to the medium to a final concentration of 0.5 mg/mL and the cells were incubated at 37°C under normoxic conditions (21% oxygen) incubator for 3 h to allow for MTT reduction. The medium was aspirated and replaced with 1 mL of dimethylsulfoxide and placed for another 15 min in incubator to solubilize the formazan crystals. Absorbance at 570 nm was measured by using a microplate reader (SpectraMax M2; Molecular Devices). The absorbance values of all Hb-incubated samples were normalized

with the absorbance values of the cells growing with the medium only. The data shown in MTT assay are relative cell viabilities expressed as means $\pm$ standard deviation (SD) of three independent experiments.

Confocal microscopy

Apoptotic and dead cells were observed using two fluorophores: AO and PI. The cells were incubated at room temperature for 15 min and permeabilized with the dyes for detection of cell viability. Fluorescent images were obtained by using the Fluoview confocal microscope (FV300, Olympus IX 81 microscope) by excitation of AO and PI at 500 and 536 nm, respectively, and emission at 530 and 620 nm, respectively. After the sequential excitation, the green and red fluorescent images of the same cells were recorded and further image analysis was done by using ImageJ software (<http://rsbweb.nih.gov/ij/>).

Statistical analysis

The statistical significance between the control group and Hb incubated formulations added to cells in hypoxic conditions were analyzed by analysis of variance to compare means of group by analyzing the estimates of variance. Multiple comparisons (differences between polymer conjugation ratios, pH effect, and antioxidants) were analyzed by two-way analysis of variance followed by Holm-Sidak test. $p < 0.05$ values was considered to be statistically significant. All the data are presented as means $\pm$ SD.

Results

Oxygen affinity of cross-linked Hb conjugates

The oxygen equilibrium curve at 37°C of isolated bovine Hb at different pH (8.0, 7.4, and 6.5) shows (Fig. 1) the fractional amount of oxygen bound. Bovine cell-free Hb has a p50 of 24.5 ± 0.6 mm Hg at normal physiological pH (7.4), close to the reported literature value of 27.4 mm of Hg.²⁵ The

FIG. 1. Fractional oxygen saturation curves of isolated free bovine Hb at different pH (8.0, 7.4, and 6.5) conditions and partial oxygen pressures. Hb, hemoglobin.

corresponding values for pH 8.0 and 6.5 were 14.05 ± 0.35 and 38.66 ± 0.62 mm Hg, respectively, which is statistically significant ($p < 0.001$). This demonstrates that oxygen level in Hb solution is higher in basic condition than in acidic conditions at any given partial pressure. The difference also suggests that if this cell-free Hb is placed in partial pressures measured in hypoxic tissues (below 10 mm Hg^{26,27} approximately between 1% and 2% oxygen), this carrier (unconjugated free Hb) is only saturated up to 30%, 16%, and 10% with oxygen at pH 8.0, 7.4, and 6.5, respectively. This shows that more oxygen is released in acidic pH environment. This also demonstrates that during hypoxia, if the pH decreases, the oxygen carrier may unload more oxygen in exchange with the carbon dioxide and protect cells by decreasing acidity. Figure 2 indicates that at pH 7.4 and 37°C, there is a statistically significant ($p < 0.001$) lowering of p50 values by PEG-2 kDa conjugation between Hb conjugates with and without antioxidant enzymes for all molar ratios when compared to p50 of cell-free bovine Hb (control). A small decreasing trend in the p50 is observed on varying the PEG ratio in the conjugates with and without antioxidant enzymes that are not statistically significant. It may also be inferred from the results that increasing the concentration of PEG-2 kDa for further lowering of p50 is not essential and an optimum

conjugation molar ratios was set. The results also show that addition of antioxidant enzymes into the conjugation mixture did not significantly alter the p50 values. More significantly, the conjugation method had decreased the p50 values of the conjugates to ~7–10 mm Hg. This lowered p50 value (high affinity) may aid in the capability of oxygen carriers to efficiently release higher amounts of oxygen in hypoxic conditions when compared to high p50 cell-free Hb.

Hill coefficient and cooperativity

Hill coefficient elucidates the cooperative nature of oxygen binding by Hb. According to the theory of oxygen binding, binding of one oxygen molecule in the heme pocket enhances the binding of subsequent oxygen molecules and thus shows the typical sigmoidal oxygen-binding curve with a positive Hill coefficient.²⁸ The values of measured Hill coefficients (Fig. 3) (n_{50} , slope measured at p50) of isolated bovine Hb molecules at 37°C are 1.76 ± 0.17 , 2.28 ± 0.10 , and 1.61 ± 0.22 at pH 7.4, 8.0, and 6.5, respectively. The positive slope values in these pH conditions show that isolated Hb exhibits positive cooperative binding. The higher the number, the better the cooperative nature of binding and Hbs typically have Hill coefficients values around 2.²⁹ The experimentally determined n_{50} values also show that Hb has higher cooperative effect in basic conditions than in acidic conditions. The results show that there is a decrease in the cooperativity of Hb molecules by this conjugation method.

The log p50 values (Fig. 4) and their corresponding n_{50} values in Hb conjugate formulations with different molar ratios of PEG-2 kDa at three different pH values and at 37°C show statistical significance ($p < 0.001$) when compared to the free Hb for all pH conditions. The n_{50} values lie in the range 1–1.5 for all conjugates without antioxidants. The increase in the amount of PEG-2 kDa for conjugation did not change the n_{50} value and thus there is an insignificant change in the cooperative nature of oxygen binding among the conjugates. Results in Figure 5 show the n_{50} values in Hb conjugated with antioxidants with a similar trend when compared to the Hb

FIG. 2. Oxygen affinity of conjugated Hb with (Hb-SOD-CAT) and without (Hb-Hb) antioxidant enzymes at Hb's p50 measured in pH 7.4. Hb-SOD-CAT, Hb-superoxide dismutase-catalase; PEG, poly(ethylene glycol).

FIG. 3. Hill plots of isolated free bovine Hb at different pH (8.0, 7.4, and 6.5) conditions.

FIG. 4. Hill coefficients (n_{50}) at corresponding log p50 of conjugated bovine Hbs (Hb-Hb) for different Hb:PEG molar ratios (1:5, 1:10, 1:15, 1:20, and 1:30) in different pH (8.0, 7.4, and 6.5) conditions.

conjugates without antioxidant enzymes. This implies that the addition of antioxidant enzymes SOD and CAT did not have any significant effect on the cooperative nature of oxygen-binding of Hb within the conjugates. Therefore, it may be feasible to adjust the antioxidant enzymes in the conjugates if a greater antioxidant effect is needed. The results show that the conjugation process did not significantly change the degree of positive cooperativity in the conjugated Hbs (with and without antioxidants). However, the positive cooperativity of Hb in Hb-Hb and Hb-SOD-CAT conjugates is decreased significantly ($p < 0.001$) when compared to free Hb. This altered cooperativity is also reflected in the altered p50 values in all the conjugates. At any given molar ratio, there is a very insignificant change in the cooperativity (n_{50}) in different pH conditions. However, increasing the molar ratio of PEG-2 kDa led to only an insignificant decrease in the Hill coefficients. This suggests that the change in cooperativity with increased PEG has no significant effect when compared to the cell-free Hb. There is a small change in log p50 and the corresponding n_{50} value with pH, so the conjugates could be less flexible to oxygen binding and release, unlike the cell-free Hb.

Bohr Effect

The Bohr Effect estimates the influence of pH on the oxygen affinity of a saturated oxy hemoglobin molecule. Bohr

FIG. 5. Hill coefficients (n_{50}) at corresponding log P₅₀ of conjugated bovine Hbs with antioxidant enzymes (Hb-SOD-CAT) for different Hb:PEG molar ratios (1:5, 1:10, 1:15, 1:20, and 1:30) in different pH (8.0, 7.4, and 6.5) conditions.

Effect is observed in most mammalian Hbs to a different degree as reflected in the Bohr coefficient. Higher the Bohr coefficient value, higher the amount of the off-loaded oxygen when the pH shifts from alkaline to acidic conditions.³⁰ The Bohr Effect originates from the conformational change of tetrameric Hb between the R (oxy) and T (deoxy) structure caused by the differences in binding and exchange between O₂ and H⁺/CO₂ at the binding sites with pH changes. There is also a change in the Hb's molecular environment of Bohr groups (specific amino acid residues). Binding of oxygen to the heme groups decreases the pK_a of the Bohr groups and makes Hb more acidic leading to release of protons.³¹ Isolated bovine Hb had a negative Bohr coefficient of 0.30 (Fig. 6) in the experiments in the tested pH range (6.5–8.0), similar to the values reported in the literature.³¹ Conjugation with PEG decreased the absolute value of Bohr coefficient by at least 50%, which is statistically significant ($p < 0.001$) compared to unconjugated free Hb. This may be due to alteration in the Bohr groups in Hbs. There was no statistically significant effect on the amount of PEG used in the crosslinking reaction mixture on Bohr coefficients. Bohr coefficient values of crosslinked Hbs show that there is only a small change in oxygen off-loading with a change in pH from 6.5 to 8. This shows that crosslinked Hbs are more susceptible to oxygen partial pressure changes than the pH changes from alkaline to acidic. Addition of antioxidant enzymes also did not

FIG. 6. Bohr plot of conjugated bovine Hbs with (Hb-SOD-CAT) and without (Hb-Hb) antioxidant enzymes at different Hb:PEG molar ratios (1:5, 1:10, 1:15, 1:20, and 1:30).

significantly change the Bohr coefficient values when compared to conjugation involving only Hb.

Oxygen content

Results (Table 1) show that the total available oxygen content is higher when p50 of Hb is lowered and calculated at different hypoxic conditions (6%, 3%, and 1%). At 40, 20, and 7 mm Hg of partial oxygen pressure; 1.2, 0.6, and 0.3 mL/L of soluble oxygen are available in the media for cellular respiration. These values are considerably low when compared to a normal partial oxygen situation of 100 mm Hg (3 mL/L). Adding free Hb molecule (0.1 mM, p50 ~ 24.5 mm Hg) to media will increase the oxygen availability by 6.7 ± 0.2 , 7.9 ± 0.2 , and 1.8 ± 0.1 mL/L at 40, 20, and 7 mm Hg of partial oxygen pressures. Crosslinked Hb with and without antioxidants will increase oxygen availability by 7.9 ± 0.2 , 6.8 ± 0.3 , and 5.2 ± 0.3 mL/L at 40, 20, and 7 mm Hg. This shows that by lowering the p50 of Hb, the percentage oxygen availability likely increases by 18%, 79%, or 189% in 6%, 3%, and 1% hypoxic conditions, respectively, and shows the importance and usefulness of the low p50 conjugate.

Viability

MTT assay estimates the cell viability by measuring the functional state of the mitochondria in the cells. Viable cells with active mitochondria convert MTT to purple crystals (formazan) in the cells, which are subsequently dissolved in organic solvents and estimated spectrophotometrically. The data (Fig. 7A, B) demonstrate that crosslinked Hb-SOD-CAT (Hb/PEG 1:10 ratio) offered better cell protection in terms of percentage relative cell viability of RINm5F cells when incubated at 37°C in hypoxic stress conditions (6%, 3%, and 1% oxygen) for 48 h than the Hb-conjugates without the antioxidant enzymes.

Percentage relative cell viabilities (Fig. 7A) in each hypoxic condition (6%, 3%, and 1% oxygen) were calculated by normalizing MTT absorbance values with control cells growing in RPMI media alone. Cell-free Hb (p50 value 24.5 mm Hg) only showed 5%–10% higher cell viability compared to cells growing in respective hypoxic conditions, suggesting poor protective effect. Crosslinked Hbs (Hb-Hb) (p50 value ~ 8 mm Hg) increased the viability by $5\% \pm 17\%$, $15\% \pm 9\%$, and $30\% \pm 13\%$ in 6%, 3%, and 1% hypoxic conditions, respectively. When Hb was crosslinked with antioxidant enzymes with a similar p50 value of 8 mm Hg, the cell viability was further increased by $32\% \pm 10\%$, $52\% \pm 12\%$, and $81\% \pm 13\%$ in 6%, 3%, and 1% hypoxic conditions, respectively. Statistical analysis showed that Hb with antioxidants offered statistically significant protection of RINm5F cells when compared to control cells with media alone ($p < 0.01$), free Hb ($p < 0.01$), and crosslinked Hb-Hb ($p < 0.05$). Crosslinked Hb with antioxidants is evidently more effective in 1% oxygen, which is relevant to islet transplantation.

When absorbance values are normalized by control cells in normoxia (21% oxygen) (Fig. 7B), cells growing in hypoxia RPMI hypoxic media alone (6%, 3%, and 1% oxygen) demonstrated decreased viabilities to $63\% \pm 11\%$, $50\% \pm 3\%$, and $45\% \pm 2\%$ in 6%, 3%, and 1% hypoxic conditions, respectively. Cell-free Hb (p50 value of 24.5 mm Hg) viabilities decreased to $66 \pm 17\%$, $57 \pm 9\%$, and $59 \pm 13\%$ in 6%, 3%, and 1% hypoxic conditions, respectively. With crosslinked Hbs (Hb-Hb) (p50 value ~ 8 mm Hg) the viability had only reduced to $83\% \pm 10\%$, $75\% \pm 12\%$, and $82\% \pm 13\%$ in 6%, 3%, and 1% hypoxic conditions, respectively.

TABLE 1. CALCULATED OXYGEN CONTENT IN HEMOGLOBIN FORMULATIONS (FREE HEMOGLOBIN; CONJUGATED Hb-Hb; CONJUGATED WITH ANTIOXIDANT ENZYMES HEMOGLOBIN-SUPEROXIDE DISMUTASE-CATALASE) AND IN MEDIA AT DIFFERENT HYPOXIC CONDITIONS (6%, 3%, AND 1% OXYGEN)

Percentage oxygen	Partial oxygen pressure	Hemoglobin formulation (0.1 mM)	Oxygen content (mL/L)	Percent increase compared to free Hb	Soluble oxygen (mL/L)	Total oxygen content (mL/L)
6%	~40 mm Hg	Hb	6.7 ± 0.2	^a	1.2	7.9
		Hb-Hb	7.9 ± 0.1	18%	1.2	9.1
		Hb-SOD-CAT	7.9 ± 0.2	18%	1.2	9.1
3%	~20 mm Hg	Hb	3.8 ± 0.1	^a	0.6	4.4
		Hb-Hb	6.8 ± 0.3	79%	0.6	7.4
		Hb-SOD-CAT	6.8 ± 0.2	79%	0.6	7.4
1%	~7 mm Hg	Hb	1.8 ± 0.1	^a	0.21	2.0
		Hb-Hb	5.2 ± 0.2	189%	0.21	5.4
		Hb-SOD-CAT	5.2 ± 0.3	189%	0.21	5.4

^aNo change or 0%.

Hb, hemoglobin; Hb-SOD-CAT, Hb-superoxide dismutase-catalase.

FIG. 7. (A) Percentage relative cell viability (MTT) of RINm5F cells incubated with RPMI media alone, free Hb, and conjugated bovine Hbs with (Hb-SOD-CAT) and without (Hb-Hb) antioxidant enzymes (Hb:PEG 1:10) for 48 h in hypoxic conditions (6%, 3%, and 1% oxygen) (values = mean $\pm$ SD; $n=3$; $*p<0.01$) MTT values are normalized by respective control cells in RPMI media and 6% or 3% or 1% oxygen. (B) Percentage relative cell viability (MTT) of RINm5F cells incubated with RPMI media alone, free Hb, and conjugated bovine Hbs with (Hb-SOD-CAT) and without (Hb-Hb) antioxidant enzymes (Hb:PEG 1:10) for 48 h in normoxia (21% oxygen) and hypoxic conditions (6%, 3% and 1% oxygen) (values = mean $\pm$ SD; $n=3$); MTT values are normalized by control cells in RPMI media and 21% oxygen. SD, standard deviation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

Confocal microscopy

The combination of acridine orange, a cell-permeable fluorescent dye that binds to DNA and emits green light when incorporated and excited in normal healthy living cells, and PI, only permeable into apoptotic or dead cells, where

the cell membrane is compromised and facilitates the incorporation of dye within the DNA and emits red light, helps distinguish qualitatively the apoptotic cells from viable cells. Figure 8 shows the confocal images of the RINm5F cells incubated with free Hb and Hb conjugates for 48 h in hypoxic conditions. Control cells growing in 6%, 3%, and 1% oxygen showed extensive cell membrane destabilization, leading to the uptake of PI by the cells. The decreased uptake of AO and the increased uptake of PI were higher in the control cells growing in 1% oxygen, indicating the decreased viability of normal cells in such harsh hypoxic conditions. This clearly demonstrated that cells incubated in hypoxic conditions without free and crosslinked Hbs show rapid cell death. The cells incubated in 6% oxygen with free and conjugated Hbs showed slightly higher cell viability (as seen in MTT assay) than control cells, but there are no significant qualitative differences in AO, and PI uptake by cells and the confocal images appear to be similar. However, the cells incubated in 3% oxygen with free Hb or conjugated Hbs without antioxidants showed significant uptake of PI, indicating cell death. The cells incubated with Hbs conjugated with antioxidant enzymes showed much higher viability. The cells growing in 1% oxygen and incubated with Hb-SOD-CAT showed cells that were mostly permeable to AO, indicating dramatic reduction of cell death. The results clearly demonstrate that Hb was effective in supplying oxygen from the conjugated Hbs in severe hypoxic environments. More significantly, it can be inferred that the low p50 cross-linked Hbs with antioxidants offered higher protection to the RINm5F cells from hypoxia-induced stress.

Discussion

The efficiency of isolated Hb-based oxygen carriers depends mainly on the proposed use of the custom-modified Hb. For designing low p50 oxygen carriers, the type of conjugating agent and conjugation chemistry needs to be carefully chosen as the method may significantly alter the sigmoidal shape of modified Hbs from the typical oxygen equilibrium curve. The commonly employed crosslinking agent reported in literature, glutaraldehyde, resulted in high p50 poly-Hb in 20:1 glutaraldehyde/Hb conjugation reaction; however, increasing the glutaraldehyde ratio to 40:1 glutaraldehyde/Hb decreased the p50 to 13 mm Hg. However, there was a significant conversion of Hb to methemoglobin (30%), thereby potentially reducing the role as an efficient oxygen carrier³² in hypoxic conditions. The PEG-driven crosslinking significantly changed the shape of OEC and thus the consequent changes in the amount of oxygen released at different partial oxygen pressures, Hill coefficient values, and Bohr coefficient values. The PEG conjugation chemistry used in our studies lowered the p50 to $\sim$ 8 mm Hg for 10:1 PEG/Hb ratios with minimal methemoglobin levels ($<5\%$),¹⁷ suggesting that these oxygen carriers may function efficiently in the intended islet cell transplantations especially in severe hypoxic conditions. It was shown in a recent study that the conjugation of PEG-chains to lysine residues of Hb by the thiolation-mediated PEGylation induced structural changes by increasing the hydration shell, which further helped in the stabilization of favorable relaxed state conformation of Hb.³³ This stabilization of relaxed state by PEG's hydration shell or modifications that help the

FIG. 8. Confocal laser scanning microscopy images of RINm5F cells incubated with (A) media only, (B) free Hb, (C) crosslinked Hb-Hb (Hb/PEG 1:10), and (D) crosslinked Hb-SOD-CAT (Hb/PEG 1:10) for 48 h in hypoxic conditions (6%, 3%, and 1% oxygen) (original magnification, $\times 100$). Color images available online at www.liebertonline.com/tea

transition of Hb from T to R conformation may be responsible for the lowering of p50.¹³ However, in the current method of conjugation, it is not feasible to know about which amino group perturbation is critical for lowering the p50 of Hb molecules and thus the lowering of the p50 of the final conjugates.³⁴ Interestingly, increasing the concentration of the PEG did not significantly lower the p50, which is contrary to our initial assumptions based on reports from the literature.³² One possible explanation may be the complete modification of amino groups³⁴ that may be involved in the structural changes in the OEC at Hb/PEG molar ratios below 1:15. The addition of antioxidant enzymes also did not significantly change the p50 of crosslinked Hb. The results also show that there is only an insignificant change in Bohr Effect and no significant changes in the oxygen release for the pH changes in the tissue or cellular environment. Experimental results also demonstrate that oxygen release by conjugated Hbs are more susceptible to hypoxic conditions (oxygen $< 6\%$) rather than the changes in cellular or media pH. The altered cooperative nature and Bohr Effect observed is common with most other polymerization techniques^{35,36} and may be attributed to transformation of Glu-101 beta to acidic Bohr group, which is in close proximity to Lys-99 alpha in oxyhemoglobin that may be involved in crosslinking.³⁷

In experiments involving hypoxic conditions, from among the different conjugates, conjugates with 1:10 (Hb/PEG) ratio were chosen for MTT viability studies. Molar ratios higher than these had no additional benefits in terms of oxygen equilibrium properties such as a low p50 value. The benefit of employing the low p50 systems in our experiments is similar to results observed by the Sakai *et al.* when being compared to high p50 systems.³⁸ Oxygen content in low p50 Hb conjugates with and without antioxidants is calcu-

lated to be similar in hypoxic conditions. However, MTT results show higher cell viability in Hb conjugate with antioxidant enzymes group. It was shown previously that PEG-conjugated Hb (MP4) incubated at 37°C for 18 h had produced 30%–40% methemoglobin as the Hb undergoes autoxidation.³⁹ The enhanced cell viability and protection from hypoxia demonstrated in our experiments can be attributed primarily to low p50 design and also protection of Hb in Hb-CAT-SOD from ROS and thus the release of higher amount of oxygen. Direct cell damage by free radicals in our experiments may be confounded with hypoxia damage and the main factor is shown to be low partial oxygen levels at 48 h interval. Only a small portion of the enhanced cell viability can be attributed to direct protection of cells from free radical damage by the antioxidant enzymes. The conversion of Hb to methemoglobin is not instantaneous and thus low p50 Hb conjugate demonstrated better viability than the free Hb in severe hypoxic conditions (1% oxygen). Despite the fact that the qualitative confocal images are similar in conjugated Hb with and without antioxidant enzymes groups, the differences in MTT cell viability may be credited to the reason that RINm5F cells in normoxia may multiply differently than hypoxia.⁴⁰

The crosslinked Hbs in our studies showed 10%–80% higher cell viability depending on the severity of hypoxia when compared to cells without any added Hb. The confocal microscopy results also support the conclusions from the MTT assay. The significant uptake of propidium iodide by control RINm5F cells when compared to cells incubated with conjugated Hbs clearly demonstrates the effectiveness of conjugated Hbs in oxygen transport in hypoxic conditions. Our previous experiments using cross-linked Hb-Hb alone had shown protection of RINm5F cells from apoptosis and

we expect similar results using Hb-SOD-CAT.⁴¹ These conjugated Hbs may also be used for enhancing the survival time of different cell or tissue types for temporary storage after isolation or during transplantation of beta cells, cardiac cells, and in ischemic conditions or related complications.^{42–44} For isolated beta cell transplantation in the low partial oxygen sites such as peritoneal cavity, exogenous low p50 Hb conjugated with antioxidants may help prevent oxygen transport issues and reduce cell death from hypoxic and free radicals stresses. Conjugated Hbs with low p50 in such situations may be more beneficial than high p50 systems or other oxygen carriers as the systems may be capable of unloading more oxygen into the cellular environment more efficiently in hypoxic conditions and thus improve the overall metabolic function.^{45–50}

Conclusion

PEG-conjugated Hbs with antioxidant enzymes provided beneficial effect of protecting cells from hypoxia, which is a unique feature of low p50 systems. Thus, this conjugation process provides useful data for optimization of O₂ carriers intended for therapeutic or prophylactic benefits from hypoxia and ischemic conditions. This study also demonstrated a general design approach for protecting isolated cells or tissues from combined hypoxia-induced oxidative and hypoxic stress.

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Disclosure Statement

No competing financial interests exist.

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