Low-Concentration Methylene Blue Maintains Energy Production and Strongly Improves Survival of Leigh Syndrome French Canadian Skin Fibroblasts

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Abstract - Leigh syndrome French Canadian (LSFC) is a recessive disease caused by mutations in the LRPPRC gene (leucine-rich pentatricopeptide repeat containing protein). These mutations induce a cytochrome c oxidase (COX) deficiency resulting in episodes of acute acidotic crisis that will often lead to death. There is no effective treatment. Methylene blue (MB) is a redox dye that increases COX content and activity *in vitro* and *in vivo* suggesting that MB could prevent and treat LSFC. In this study, the protective effect of low-concentration MB was tested on two LSFC cell lines, including LSFC-F1, homozygous for the mutation A354V, and LSFC-F2 a compound heterozygous for the mutations A354V and C1277STOP. MB effect on metabolic activity was assessed on both LSFC cells in stable and acidotic conditions. For LSFC-F1, results showed that metabolic activity drastically decline after 96 hours in both conditions but not LSFC-F2 and normal cells. MB completely prevents the decrease of metabolic activity in LSFC-F1. Intracellular ATP content was also measured in both culture media. After 96 hours in acidotic medium, ATP content was almost completely depleted for both LSFC cells. Interestingly, MB completely restores ATP content in LSFC-F1 and LSFC-F2 cells. Finally, MB strongly improves the survival of both LSFC cells.

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INTRODUCTION

The French Canadian variant of Leigh syndrome (Leigh Syndrome French Canadian, or LSFC), also commonly called lactic acidosis, is a recessive disease caused by mutations in the LRPPRC gene (leucine-rich pentatricopeptide repeat containing protein) on chromosome 2p16-21. One mutation (Ala^{354} → Val) [C^{1119} → T] was found responsible for 95% of the cases of LSFC in the Saguenay-Lac-St-Jean region (1, 2). One individual had an 8bp deletion in exon 35 creating a premature stop codon thus producing one compound heterozygote. Children who suffer from this illness are hypotonic and have psychomotor delay (3). Classical Leigh syndrome presents with typical characteristics such as ataxia, dystonia, optic nerve atrophy, ophthalmoplegia, ptosis, nystagmus, trembling and respiratory system alterations. However, the LSFC symptoms are less severe than those observed in the "classical" Leigh syndrome (3). In LSFC, energy production is altered since the cells of children suffering from this disease produce less energy (ATP) than normal cells (4). This reduced energy production is caused by a deficit in cytochrome c oxidase (COX), an enzyme involved in the electron transfer chain of mitochondria (4). Recently, Sasarman et al., showed that the level of mutated LRPPRC is reduced in LSFC cells resulting in decreased steady-state levels of most mitochondrial mRNAs and proteins which lead to a COX assembly defect (5). In a normal, unstressful situation, afflicted children can overcome this deficiency. However, a sustained physical effort, an emotional shock or an infection can all prove fatal.

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These stresses may result in an acute acidotic crisis with excessive accumulation of lactic acid that leads to coma and death (3). Many tissues isolated from LSFC children express low COX activity, noticeably in the brain and liver (10 to 20% of the normal activity) (4). Some tissues, such as skin (fibroblasts) and skeletal muscle, maintain 50% of their normal COX activity, while tissues such as heart and kidney show almost normal levels of COX activity (4). To date, there is no effective treatment available.

The redox agent methylthioninium chloride or methylene blue (MB) is an organic dye from the diaminophenothiazine family with cationic and lipophilic properties that acts as an electron transfer mediator in the mitochondria (6-9). Atamna et al. (9) demonstrated that nanomolar range of MB delays cellular senescence of normal human fibroblasts and increases oxygen consumption, heme synthesis and resistance to oxidative damage. Interestingly, MB could also increase the cellular contents of cytochrome c oxidase. The promising properties of MB prompted us to evaluate its potential on cells affected by LSFC.

The objective of this study was to evaluate the protective effect of MB on two skin fibroblast cell lines isolated from LSFC patients. The effect of MB on metabolic activity, ATP content and cell survival was assessed with regard to two culture conditions, i.e. a stable and an acidotic media. The results showed that MB maintains metabolic activity, ATP content and improves the survival of two LSFC cells tested.

Materials and Methods

Primary explant and cell culture
The normal human skin fibroblast cell line, WS1 (ATCC #CRL-1502) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The LSFC cell lines were obtained - after informed consent - from skin biopsies of two patients, using primary explants (10). A pure skin fibroblast cell line was obtained from each patient. The LSFC cell lines were named LSFC-F1 for the homozygous (A354V) and LSFC-F2 for the compound heterozygous (A354V and C1277STOP). The cell lines were grown in Dulbecco's Modified Eagle's Medium (Mediatech Cellgro®, Herndon, USA). The culture medium was supplemented with 10% fetal calf serum (Hyclone, Logan, USA); a solution of vitamins containing : D-calcium pantothenate (1 mg/L), choline chloride (1 mg/L), folic acid (1 mg/L), inositol (2 mg/L), Nicotinamide (1 mg/L), Pyridoxine (1 mg/L), Riboflavin (0.1 mg/L), Thiamine (1 mg/L); sodium pyruvate (10 mM); non-essential amino acids solution containing : L-alanine (8.9 mg/L), L-asparagine (15 mg/L), L-aspartic acid (13.3 mg/L), L-glutamic acid (14.7 mg/L), glycine (7.5 mg/L), L-proline (11.5 mg/L), L-serine (10.5 mg/L); penicillin (100 IU) and streptomycin (100 μg/ml) (Mediatech Cellgro®). Cells were cultured in a humidified atmosphere at 37 ºC in 5% CO_2. This study was approved by the Comité d'éthique à la recherche de l'Université du Québec à Chicoutimi and the Comité d'éthique à la recherche du Complexe hospitalier de la Sagamie.

Assessment of COX activity
Isolated mitochondria were prepared as described by Pitkänen et al. (11). Protein concentration of the mitochondrial fraction was determined using Bradford's method with BSA as a standard (12). The cytochrome c oxidase (COX) and citrate synthase (CS) activities were measured as described by Kramer et al. (13) at the same protein concentration. The COX activity was expressed as COX/CS ratio.

Methylene blue treatment
Methylene blue, MB (chemical grade; purity ≥ 95%), was obtained from Sigma-Aldrich (St-Louis, MO, USA). The cells were treated with or without 125 nM MB during 24 or 96 hours in a stable medium containing Earl's Salt medium base, 4 mM glucose, 4 mM lactate, 18 mM sodium bicarbonate at a pH adjusted to 7.3 or in an acidotic medium containing Earl's Salt medium base, 30 mM glucose, 10 mM lactate, 7 mM sodium bicarbonate at a pH adjusted to 7.0.

Metabolic activity using resazurin
Exponentially growing cells were plated at a density of 1×10^4 cells per well in 96-well microplates (Costar, Corning Inc.) in 100 μl of culture medium and were allowed to adhere for 24 hours before treatment. The cells were treated with or without 125 nM MB in a stable medium or an acidotic medium. After 24 h or 96 h, 10 μl of a solution of resazurin 4% was added to each well and the plates were incubated 60 minutes at 37ºC

(14). Fluorescence was measured on an automated Fluoroskan Ascent Fl plate reader (Labsystems) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The metabolic activity was reported on the relative quantity of DNA found in each well using Hoechst assay (15). Briefly, the medium was removed from the wells. The cells were washed with PBS buffer, pH 7.4 (Sigma-Aldrich, St-Louis, MO, USA) and the plates were frozen at -80ºC for 24 hours. The plates were then thawed to room temperature and shaken for three hours with 100 µL of SDS 0.01 % per well. The plates were again frozen for 24 hours at -80ºC and thawed to room temperature. Then, we added to each well 100 µL of solution containing 60 ng/mL Hoechst 33342 in TNE buffer (10 mM Tris-HCl, 1 mM EDTA, 4 M NaCl) adjusted to pH 7.4. The plates were shaken for 2 hours at room temperature in the dark. Fluorescence was then measured (ex.: 460 nm; em.: 530 nm). Regardless of the conditions tested (n>30), the mean of relative Hoechst fluorescence for WS1, LSFC-F1 and LSFC-F2 was 8 ± 1, 2.0 ± 0.4 and 2.6 ± 0.5, respectively.

Assessment of ATP content

Exponentially growing cells were plated at a density of 10^4 cells per well in 96-well microplates (Costar, Corning Inc.) in 100 µl of culture medium and were allowed to adhere for 24 hours before treatment. Then, the cells were treated with or without 125 nM MB in a stable medium or an acidotic medium for 24 or 96 hours. The cells were washed with PBS and 100 µl of boiling distilled demineralized water was added to the cells (16). Five microliters from each sample was added to an opaque microplate. Using an automated system, 45 µL of a reaction solution (D-luciferin 0.5 mM; DTT 1 mM; firefly luciferase 1.25 µg/mL) was injected and light emission was measured immediately over 10 seconds. ATP content of samples was determined using standard curves with known concentrations of ATP and expressed as ATP/Hoechst ratio.

Survival assay using calcein-AM

Exponentially growing cells were plated at a density of 10^4 cells per well in 96-well microplates (Costar, Corning Inc.) in 100 µl of culture medium and were allowed to adhere for 24 hours before treatment. Then, the cells were treated 96 hours

with or without 125 nM MB in a stable medium or an acidotic medium. The viability of cells was assessed by the presence of intracellular esterase activity using calcein-AM (Invitrogen, Burlington) (17). The probe was diluted at a final concentration of 4 µM and added directly to the cells. The plates were incubated at 37ºC with 5% CO_2 for 2 hours and fluorescence (ex.: 485 nm; em.: 530 nm) was read using a Fluoroskan Ascent FL plate reader (Labsystems).

STATISTICAL ANALYSIS

In all studies the values were expressed as means ± standard deviation of six determinations. The distribution of each group was found to be normal by using the Kolmogorov-Smirnov test ($p > 0.200$). The results were analyzed by one-way ANOVA followed by a post-hoc Bonferroni test. P values of 0.05 or less were considered statistically significant.

RESULTS

Cytochrome oxidase activity of Leigh Syndrome French Canadian skin fibroblasts.

COX activity was evaluated on isolated mitochondria. Results presented in Figure 1 are expressed as the ratio of COX activity on citrate synthase (CS) activity made on the same quantity of proteins. The COX/CS ratio for normal skin fibroblasts (WS1) and Leigh Syndrome French Canadian skin fibroblasts, LSFC-F1 and LSFC-F2, was 1.0 ± 0.2, 0.47 ± 0.05 and 0.5 ± 0.1 respectively. The COX activity for both LSFC cells was significantly lower, i.e. about 52% in comparison with normal fibroblast WS1. No significant difference was found between LSFC-F1 and LSFC-F2. These results agree with those reported in the literature (4).

Effect of methylene blue on metabolic activity and ATP content of Leigh Syndrome French Canadian skin fibroblasts.

The effect of MB on the metabolic activity of WS1 and LSFC cells was evaluated using resazurin in two culture media with regard to physiological blood concentration of glucose, lactate, bicarbonate and pH measured in LSFC patients before (stable medium) and during the acidotic crisis (acidotic medium). Optimal concentration of MB was firstly

determined in acidotic medium. LSFC cells were incubated in the presence or absence of growing concentrations of MB ranging from 0,016 to 10 µM for 24 and 96 hours. After 24 hours, the results presented in Figure 2 show that MB does not affect significantly the metabolic activity of LSFC cells. After 96 hours in acidotic medium, a decrease of 77% of the metabolic activity was observed in comparison with 24 hours. MB significantly increases metabolic activity between 0.031 and 1 µM with a maximum at 0.125 µM. However, MB induced significant cytotoxicity at concentrations of 5 and 10 µM. Consequently, the optimal concentration of MB used in this study was 0.125µM (125 nM). The possibility that MB directly interacts with resazurin was investigated. The effect of pH was also tested. Resazurin was incubated, without cells, for 1 hour in the absence or in the presence of MB 125 nM at pH ranging from 5.5 to 7. The basic fluorescence at time 0 was compared with fluorescence after 1 hour of incubation. The results indicate that MB does not react directly with resazurin (data not shown). Moreover, the results show also that pH does not promote direct reaction of MB with resazurin (data not shown). Consequently, the effects of MB are induced by biological effects.
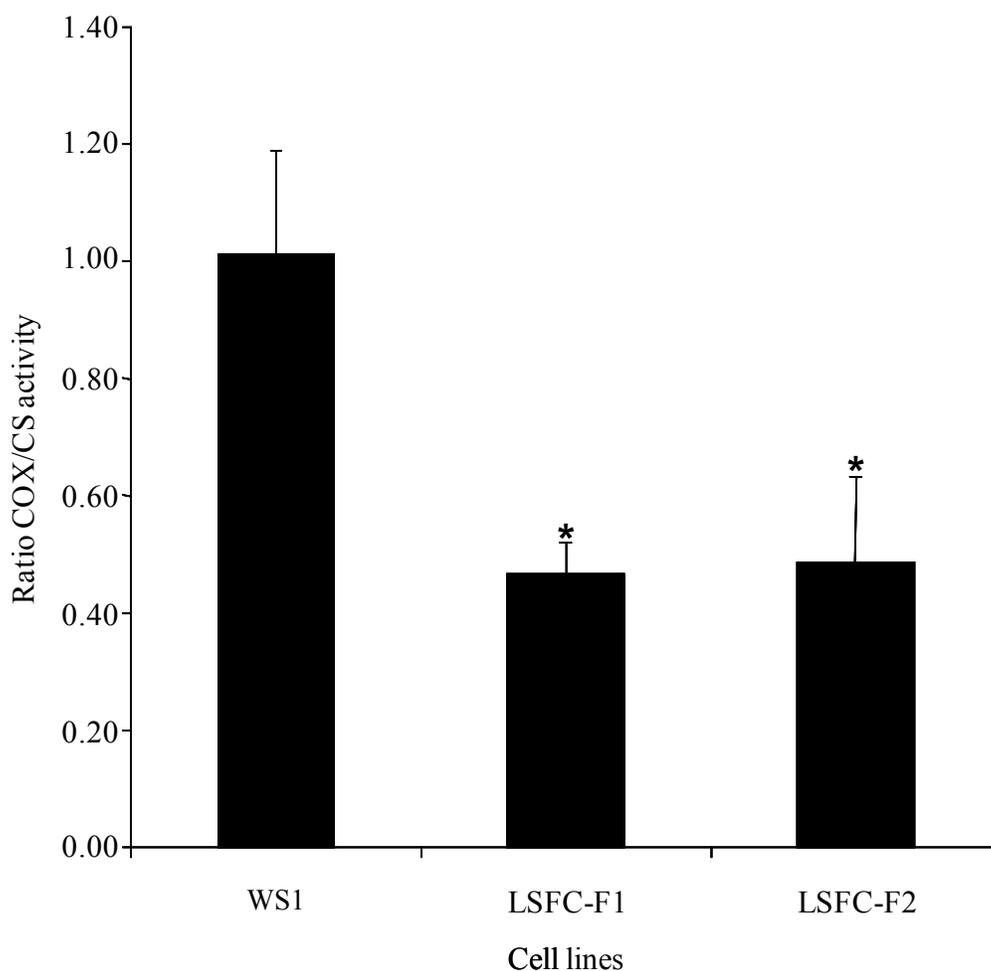

Fig. 1 Cytochrome c oxidase activity of normal (WS1) and Leigh Syndrome French Canadian (LSFC) skin fibroblasts. Data represent the means ± standard deviation of six determinations. *Significantly different from normal skin fibroblasts WS1; P ≤ 0.05, one-way ANOVA analysis and post-hoc Bonferroni test.
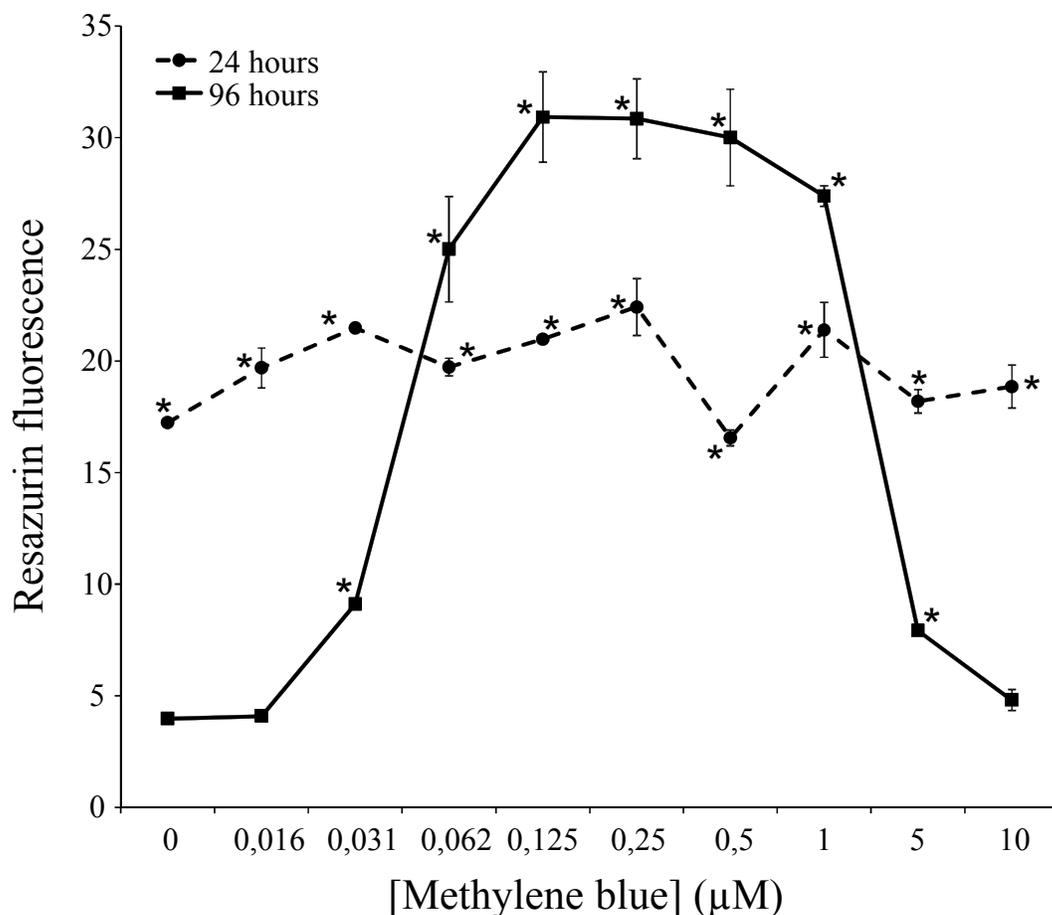
Fig. 2 Concentration-response curve of LSFC cells treated with or without methylene blue (0.016 to 10 μM) in acidotic medium for 24 and 96 hours. Data represent the means ± standard deviation of at least six determinations. *Significantly different from MB-untreated cells at 96 h. $P \leq 0.05$, one-way ANOVA analysis and post-hoc Bonferroni test.

To compare the effect of MB on the metabolic activity of normal and LSFC skin fibroblasts, the cells were treated with or without 125 nM MB for 24 and 96 hours in a stable medium or an acidotic medium. The metabolic activity was reported on the relative quantity of DNA found in each well. Results presented in Figure 3 show that after 96 hours the metabolic activity of WS1 significantly increased by approximately 43% in a stable medium and by approximately 76% in the acidotic medium in comparison to 24 hours. However, no change was observed for LSFC-F2. In contrast, a drastic decrease of metabolic activity was observed for LSFC-F1 after 96 hours in both media. The decline of the metabolic activity is completely prevented by MB in the stable medium (Figure 3A)

and the acidotic medium (Figure 3B). Moreover, MB significantly increases the metabolic activity of LSFC-F2 after 96 hours in the acidotic medium in comparison with untreated cells. However, MB has no significant effect on metabolic activity of LSFC-F2 after 24 hours and on WS1 after 24 and 96 hours. On the other hand, it is interesting to note that, after 24 hours in the stable medium (Figure 3A) the metabolic activity of LSFC-F1 is significantly higher, about 130% in comparison with normal cells. Moreover, after 24 h in the acidotic medium (Figure 3B), the metabolic activity of both LSFC cell lines is higher in comparison with WS1. Altogether, these results suggest that MB could prevent the decline of metabolic activity and protect energy homeostasis in LSFC cells.

Therefore, the effect of MB on ATP content was assessed on both LSFC cells and compared with WS1. ATP content was reported on the relative quantity of DNA found in each well. The results presented in Figure 4 show that, at 24 hours, the ATP content is about 40% to 60% lower in both LSFC cell lines in comparison with WS1 in the stable (Figure 4A) and acidotic media (Figure 4B). After 96 hours, the ATP content of LSFC-F1 was significantly decreased by approximately 73% in the stable medium and by approximately 97% in the acidotic medium compared with untreated cells at 24 hours. The treatment with MB completely prevents the decline of ATP in both media. As observed in WS1, the ATP content in LSFC-F2 did not decrease in the stable medium after 96 hours (Figure 4A). In contrast, a significant decrease was observed after 96 hours for WS1 and LSFC-F2 in the acidotic medium (Figure 4B). The decline of ATP is more marked for LSFC-F2 with a decrease of 91% compared with a decrease of 53% for the WS1. Interestingly, MB completely restores the ATP content in LSFC-F2 but not in WS1. Altogether, these results show that MB can maintain ATP content in both LSFC cell lines and suggest that MB could allow the improvement of cell survival.

Effect of methylene blue on survival of Leigh Syndrome French Canadian skin fibroblasts.
To assess the effect of MB on cell survival, the LSFC-F1, LSFC-F2 and WS1 cells were treated with or without 125 nM MB for 24 and 96 hours. The percentage of survival of cells at 96 hours was calculated from calcein fluorescence at 24 hours which is considered as 100% of survival. Results presented in Figure 5 show that the percentage of survival of WS1, ranging from 54% to 69%, was not significantly different between the two culture media. Moreover, MB did not improve cell survival. Interestingly, the survival of LSFC-F2 is significantly higher than normal cells in the stable medium with 82% but not in the acidotic medium with 40% of survival. The MB treatment of LSFC-F2 increases cell survival in the stable and acidotic medium to approximately 27% and 54% respectively compared to untreated cells. Finally, LSFC-F1 cells did not survive in the two culture media after 96 hours. However, MB strongly improves the survival of LSFC-F1 in both media with 82% and 99% of survival, respectively.

Altogether, these results indicate that MB strongly improves the survival of both LSFC cells.

Discussion

LSFC disease is characterized by COX deficiency which can result in acidotic crises and lead to coma and death (3,4). Some studies show that MB increases COX content and activity suggesting that MB could prevent acidotic crises in LSFC disease (9,18). MB effect was evaluated on a spatial memory retention deficit in rats induced by a COX inhibitor, sodium azide. MB was shown to increase mitochondrial respiration at low doses ranging from 0.1 to 2 µM, transferring electrons from complex IV to oxygen (6,9,19,20). Interestingly, administration of a low dose of methylene blue (1 mg/kg) in rat completely restored the memory retention impaired by the inhibitor of COX, thus suggesting that MB can compensate inhibited mitochondrial respiration in brain (20). Moreover, Callaway et al. (18) reported that MB improves cognitive functions in normal rats by increasing the brain cytochrome c oxidase activity. Recently, Atamna et al. (9) demonstrated that nanomolar range of MB delays cellular senescence of normal human fibroblasts and increases oxygen consumption, heme synthesis and resistance to oxidative damage. Interestingly, MB could also increase the cellular contents of cytochrome c oxidase.

The objective of this study was to evaluate *in vitro* the protective effect of MB on LSFC cells in two culture conditions as stable and acidotic media. COX activity in LSFC disease is variable according to the tissues (4). The most important decreases are observed in brain, liver and lungs with only 10 to 20 % of the normal COX activity (4). However, cultures of LSFC cells of brain, liver or lungs are not available to evaluate the potential of drugs such as methylene blue. Moreover, there is no animal model. In a previous work, Merante et al. (4) reported that the COX activity of fibroblasts isolated from LSFC patients, cultivated in normal conditions, was decreased about fifty percent in comparison with normal skin fibroblasts. Given that skin fibroblasts are much easier to obtain, this model was chosen for the present study. As reported in the literature (4), COX activity in both LSFC cells was reduced about fifty percent in comparison with normal skin fibroblasts, WS1.
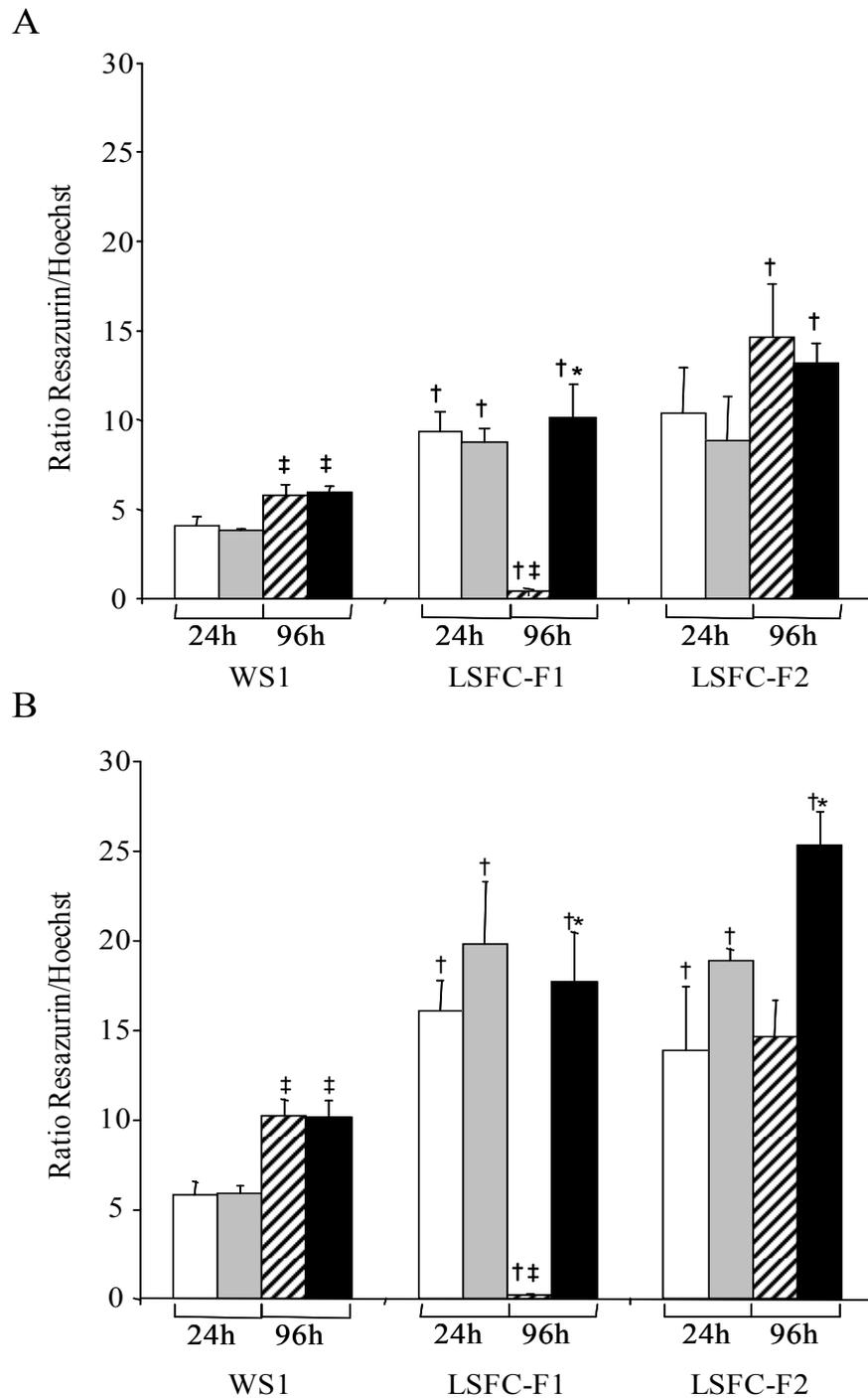
Figure. 3 Effect of methylene blue on the metabolic activity of normal (WS1) and LSFC skin fibroblasts in stable medium (A) and acidotic medium (B). The cells were incubated for 24 h (white) or 96 h (dashed) in the absence or in the presence of 125 nM methylene blue (gray & black). Data represent the means ± standard deviation of at least six determinations. Statistical analysis was performed independently for each cell type in each medium. LSFC cells were also compared with normal skin fibroblasts according to the time and the treatment. *Significantly different from MB-untreated cells for LSFC-F1 or LSFC-F2. ‡Significantly different from cells at 24 h for WS1 or LSFC-F1. †Significantly different from normal skin fibroblasts WS1 with regard of time and treatment for LSFC-F1 or LSFC-F2. P ≤ 0.05, one-way ANOVA analysis and post-hoc Bonferroni test.
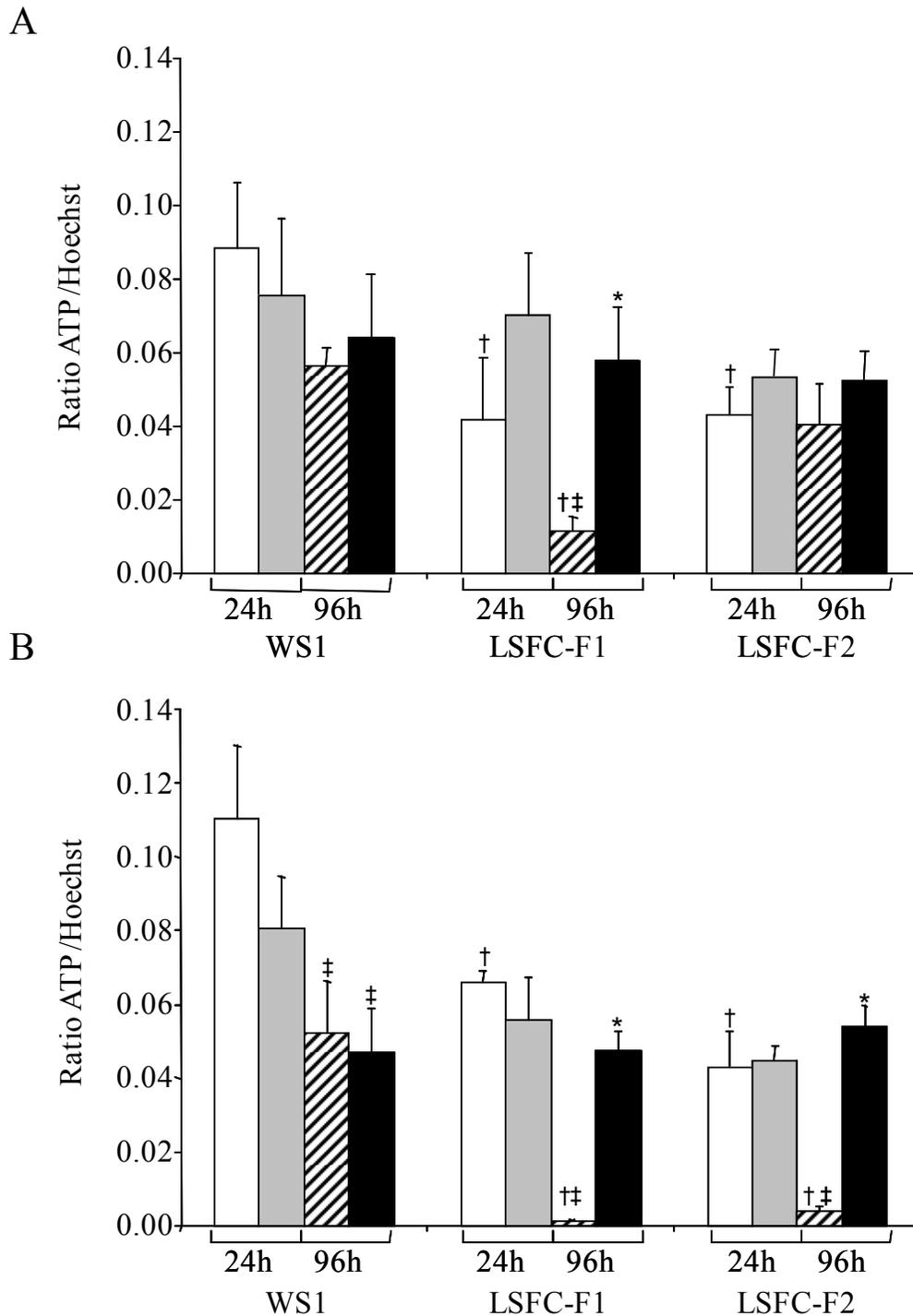
Figure. 4 Effect of methylene blue on the ATP content of normal (WS1) and LSFC skin fibroblasts in stable medium (A) and acidotic medium (B). The cells were incubated for 24 h or 96 h in the absence (white-) or in the presence of 125 nM methylene blue (gray & black). Data represent the means ± standard deviation of at least six determinations. Statistical analysis was performed independently for each cell type in each medium. LSFC cells were also compared with normal skin fibroblasts according to the time and the treatment. *Significantly different from MB-untreated cells for LSFC-F1 or LSFC-F2. ‡Significantly different from cells at 24 h for WS1 or LSFC-F1. †Significantly different from normal skin fibroblasts WS1 with regard to time and treatment for LSFC-F1 or LSFC-F2. P ≤ 0.05, one-way ANOVA analysis and post-hoc Bonferroni test.
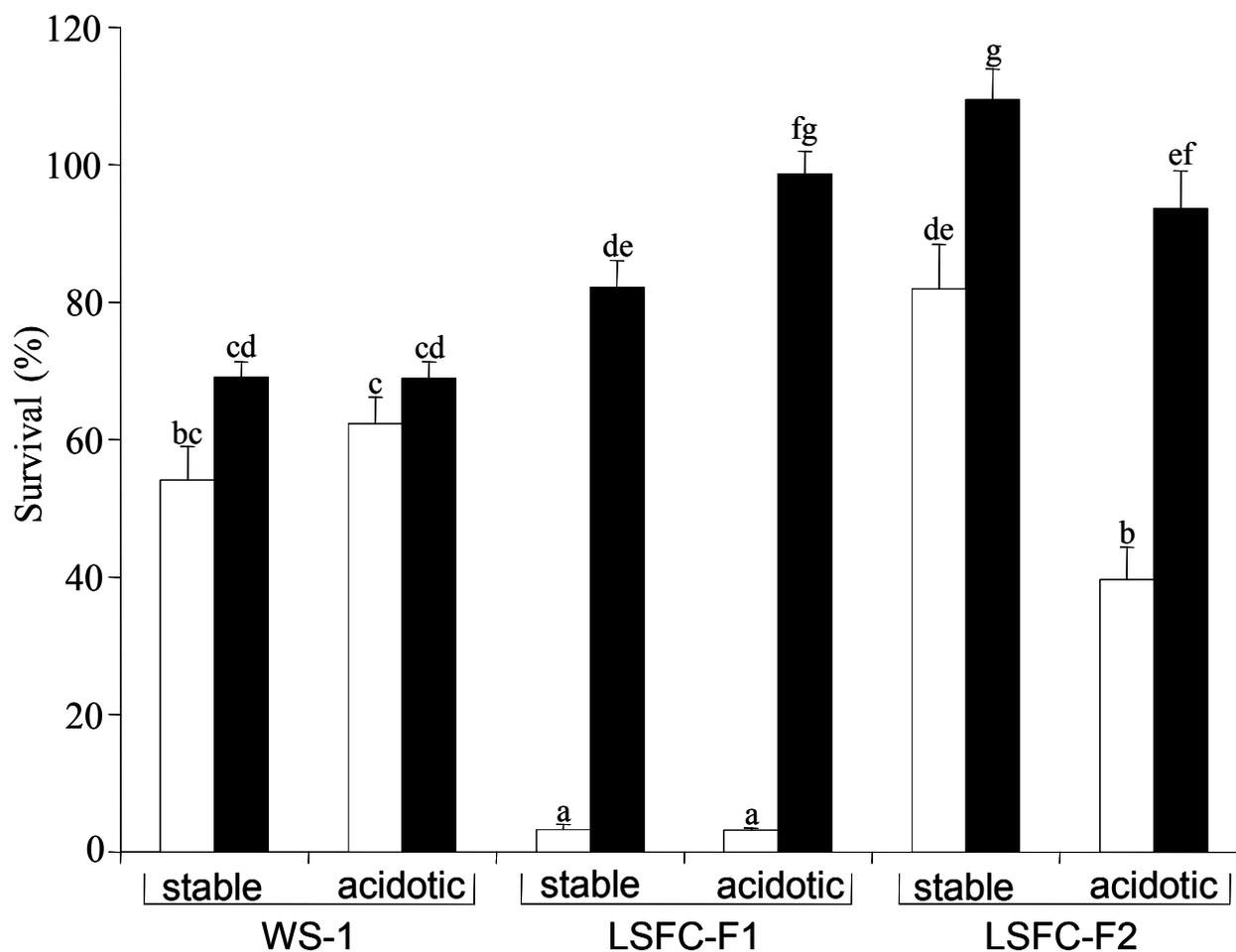
Figure. 5 Effect of methylene blue on the survival of normal and LSFC skin fibroblasts. Each cell line was incubated for 96 hours in stable medium or acidotic medium with (black) or without (white) 125 nM methylene blue. Data represent the means ± standard deviation of at least six determinations. Means within each group with different letters (a-g) differ significantly from each others. P ≤ 0.05, one-way ANOVA analysis and post-hoc Bonferroni test.

Using these cellular models, we tested the effect of MB on metabolic activity, ATP content and survival in two culture conditions as stable and acidotic media.

Metabolic activity was measured using resazurin which is a redox indicator dependent on cellular reduced equivalents such as NADH and NADPH. Non-fluorescent resazurin is irreversibly reduced to fluorescent resorufin by NADH-dependent oxido-reductase (21). The reduction of resazurin is shown to be dependent on both glycolytic and oxidative metabolism (22). Therefore, resazurin was used to determine general metabolic activity including mitochondrial function (23-26). Interestingly, our results show that the

metabolic activity at 24 hours was found generally higher in both LSFC cell lines in comparison with normal cells. This observation could be due, in part, to the accumulation of reduced equivalents as NADH possibly caused by a decreased COX activity and sustained glycolysis (4). The NADH/NAD+ ratio is implicated in the regulation of glucose metabolism. Therefore, elevated NADH/NAD+ ratio can cause a blockage of glucose oxidation and reduce the ATP content (27,28). Moreover, the NADH accumulation can lead to the transformation of the pyruvate to lactate through lactate dehydrogenase (29). Intracellular acidification induced by lactic acid could be unfavourable to the preservation of COX activity

and this effect may be exacerbated in LSFC cells. After 96 hours in both media, our results indicate that metabolic activity and ATP content were drastically reduced in LSFC-F1. Surprisingly, the compound heterozygous LSFC-F2 was found to be unaffected after 96 hours in the stable medium compared with the acidotic medium and with homozygous LSFC-F1 cells. Moreover, LSFC-F2 was found to be more resistant in the stable medium than normal cells and LSFC-F1 cell lines and more resistant in the acidotic medium compared with LSFC-F1. It is interesting to mention that the compound heterozygous patient survived about twelve acidotic crises. LSFC-F2 resistance could be explained, in part, by a COX VIc polymorphism that replaces an acidic glutamate 62 with a glycine 62 (1). Indeed, this polymorphism possibly decreases the sensitivity of LSFC-F2 cells in acidic conditions avoiding such a decline of the COX activity. This hypothesis may give a physiological clue to the patient's crises. Indeed, that a stress perhaps temporarily lowers the pH by increasing lactate production and this then lowers the ability of COX to maintain its activity. Testing of this hypothesis is in progress.

Preliminary experiments allowed the determination of the optimal MB concentration at 125 nM (Figure 2). It is important to mention that relatively high concentrations of MB (>1 μM) are cytotoxic for normal and LSFC cell lines (data not shown). These results are in agreement with the literature (9). Our results show that MB prevents decline of metabolic activity as well as maintain redox state and ATP content in both LSFC cells. Moreover, MB improves survival of LSFC cells in both media. It was reported that MB+ can oxidize directly NADH into NAD+ (19) and the MBH2 produced can be re-oxidized directly by molecular oxygen (29-31). Decrease of NADH/NAD+ ratio by MB could prevent intracellular acidification and the inhibition of the glycolysis and possibly oxidative metabolism including COX activity. Moreover, in the cells MB+ could also act as an acceptor of hydrogen in several enzymatic systems (33,34), thus favouring the reactions of MBH2 with the heme group of proteins (32,35). As proposed by Atamna et al. (9) in IMR90 lung fibroblasts, it is possible that MB+ and MBH2 could serve as electron carriers between NAD(P)H-dependent dehydrogenase of mitochondrial complex I and the heme groups of COX. The MB+/MBH2 redox-

cycling in mitochondria of LSFC cells could increase COX activity, consumption of oxygen and maintain ATP content. On the other hand, MB might also increase COX components of mitochondrial complex IV in LSFC cells as shown by Atamna et al. (9) in IMR90 lung fibroblasts. However, complementary studies will be necessary to confirm these hypotheses. Finally, in a previous clinical study, the treatment of five patients with intravenous MB had a demonstrable effect on all five patients, resulting in a lowering of the lactate/pyruvate ratio, a pronounced decrease of excess lactate, and may have contributed to the survival of one patient (33).

In conclusion, the results presented in this study show that a very low concentration of MB can prevent the decline of metabolic activity in LSFC cells and the decrease of ATP content. Moreover, MB was found to strongly improve the survival of LSFC cell lines suggesting that it could prevent acidotic crises. The fundamental decompensation in acidotic crises of LSFC patients is a frequent cause of fatality and thus any manipulation with drugs that can defray the cytotoxic events in crisis could be enormously advantageous. To the best of our knowledge, MB therapy was never tested on lactic acidosis induced by inherited metabolic disorders such as LSFC. It would be interesting to investigate the protective effect of MB at very a low dose in LSFC disease *in vivo*.

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