

Time-dependent effects of the promising anticancer drug 3-bromopyruvate and its biochemical analogs pyruvate and lactate (Warburg effect) on C6 glioma-induced H₂O₂ production

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Abstract

Background: Pyruvate (an antioxidant) result from aerobic glucose oxidation (aerobic glycolysis) while lactate (a pro-oxidant metabolite in the tumor microenvironment) results from anaerobic glycolysis. Lactate is a structural analog to pyruvate (formed in anaerobic conditions via adding two hydrogen atoms to pyruvate carried on the coenzyme NADH.H) via the enzyme lactate dehydrogenase. Both pyruvate and lactate are structural analogs of the promising anticancer drug 3-bromopyruvate (3BP). 3BP acts via competing with lactate and pyruvate.

Objectives: To investigate if H₂O₂ production is induced by C6 glioma cells and added 3BP at different time points and the effects of serial doses of exogenous lactate and pyruvate on H₂O₂.

Materials and methods: C6 glioma cells were cultured till reaching confluency and fresh medium containing the scheduled treatments was added. H₂O₂ was assayed (30, 45, 60 and 120 minutes later).

Results: The author confirmed his previous findings that 3BP significantly induced H₂O₂ production (that persisted over these different time points) (p<0.001) compared to control. C6 glioma cells significantly induced the formation of H₂O₂ (measured in RFU) at the same different time points. H₂O₂ induced by the cancer cells was still significantly high (more than the baseline values upon adding serial doses of lactate). Serial doses of pyruvate significantly and maximally scavenged glioma-induced H₂O₂ production (p<0.001). Serial doses of lactate did not decrease H₂O₂ below the baseline values i.e. exogenous lactate did not scavenge H₂O₂.

Conclusion: C6 glioma cells induce the formation of H₂O₂ at baseline and this decreased with adding the new culture medium at different sequential time points. 3BP induced the generation of H₂O₂ that persisted for at least two hours. Serial doses of lactate did not scavenge C6 glioma-induced H₂O₂ production. Serial doses of pyruvate significantly and maximally scavenged C6 glioma-induced H₂O₂ production.

Keywords: Lactate; Pyruvate; C6 glioma; H₂O₂; 3-bromopyruvate.

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Introduction

Pyruvate (an antioxidant) result from aerobic glucose oxidation (aerobic glycolysis) while lactate (a pro-oxidant) results from anaerobic glycolysis. Lactate is a structural analog to pyruvate and formed from pyruvate in anaerobic conditions via adding two hydrogen atoms to pyruvate (carried on the coenzyme NADH.H) through the activity of the enzyme lactate dehydrogenase (El Sayed et al., 2013; El Sayed, 2018, 2023). Lactate was reported as an epigenetic metabolite that drives survival in model systems of glioblastoma (Torrini et al., 2022) and reversing the Warburg effect was suggested as a treatment for glioblastoma. Like the majority of malignancies, glioblastoma multiforme (GBM) has an uncommon aerobic glycolysis bioenergetic state known as the Warburg effect. In GBM cell lines, the Warburg effect is reversed by methylene blue. This is demonstrated by increased oxygen consumption, decreased lactate production, decreased GBM cell proliferation, stopped cell cycle in S phase, and decreased cyclin expression. In GBM cell lines that are both temozolomide-sensitive and -insensitive, methylene blue suppresses cell growth. (Poteet et al., 2013).

According to Otto Warburg's 1927 description of the role of lactate in cancer,

when cancer cells take in large amounts of glucose, they produce a noticeable amount of lactate—a phenomenon known as the "Warburg effect." Since then, lactate has emerged as a key signaling molecule involved in the development of cancer. When it is released from tumor cells, the tumor microenvironment (TME) becomes acidified, with an acidification range of 6.3 to 6.9. This encourages activities including tumor promotion, angiogenesis, metastasis, tumor resistance, and, most critically, immunosuppression, which has been linked to a bad prognosis (Pérez-Tomás and Pérez-Guillén, 2020). High expression of glycolytic genes in clinical glioblastoma patients correlates with decreased patients' survival (Stanke et al., 2021). Both lactate and pyruvate are structural analogs of the promising anticancer drug 3-bromopyruvate (3BP) (Fig.1). In the author's earlier studies, our published data confirmed that 3BP works (at least partially) through antagonizing both lactate and pyruvate and C6 glioma cells (that act as a model for studying the behavior of glioblastoma tumors both in vitro and in vivo) can produce a significant amount of hydrogen peroxide (El Sayed et al., 2012a; El Sayed et al., 2012b).

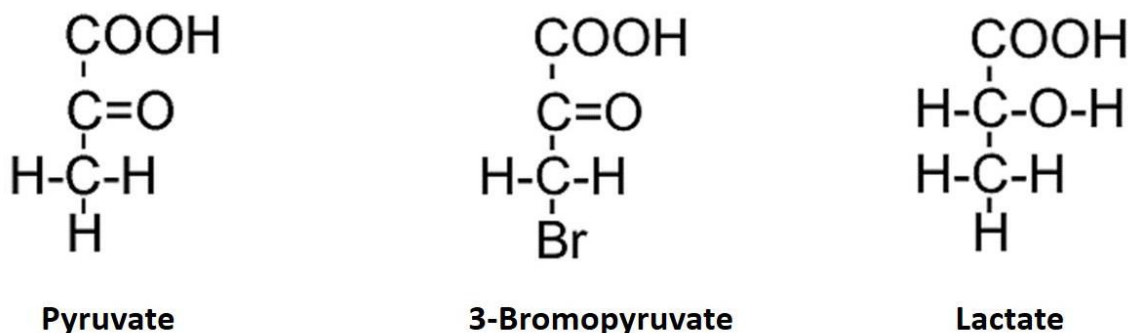


Fig.1. Pyruvate and lactate are two structural analogs of the promising anticancer drug 3-bromopyruvate.

One of the most important metabolites in the tumor microenvironment is lactate. Lactate's physiological concentrations in blood and healthy tissue are between 1.5 and 3 mM. High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers (Walenta et al., 2000). Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer. Extracellular quantities of lactic acid can reach up to 40 mM when released by tumor cells (Brizel et al., 2001). Fisher et al. in 2017 reported Inhibitory effects of tumor cell-derived lactic acid on human T cells and their study provided evidence for this, as they discovered elevated lactate levels in the sera of individuals suffering from a variety of malignancies, including melanoma, sarcoma, lung, urogenital, and breast cancers (Fischer et al., 2007). The hypoxia, which results from the unbalanced angiogenesis that characterizes the majority of tumors, is another significant physiological aspect of the tumor microenvironment. Changes in cellular metabolic pathways brought about by hypoxia include an increased reliance on aerobic glycolysis, which raises lactate generation when combined with glutaminolysis (Vaupel and Mayer, 2014).

Endogenous H_2O_2 production was reported in cancer cells to increase its steady-state oxidative stress condition. Extracellular H_2O_2 released from human liver cancer cells was detected based on TiO_2 nanoneedles with enhanced electron transfer of cytochrome c (Luo et al., 2009). Endogenous H_2O_2 was also reported in C6 glioma cells cultured in DMEM medium and significantly increased upon adding 3BP treatment (El Sayed et al., 2012a; El Sayed et al., 2012b). This study sheds light on the time-dependent H_2O_2 production by glioma

cells compared to the time-dependent H_2O_2 production induced by 3BP.

Materials and methods

In vitro C6 glioma culture

According to the manufacturer's protocols, C6 cells were seeded into 96-well plates at a density of 1×10^4 cells per well. After adding a new medium containing either 3BP, serial doses of pyruvate or serial doses of lactate. The cells were allowed to culture for an additional hour. Following this, the medium was removed and washed once more in 50 milliliters of phosphate-buffered saline solution.

H_2O_2 assay

Using the Amplex red kit (Molecular Probes), RFU readings were detected. H_2O_2 produced from all the treatment conditions (60 μ M 3BP, serial doses of lactate and serial doses of pyruvate) were measured using the fluorescence function of the multimode microplate reader Biotek Synergy (VT, USA). RFU has been detected using the Amplex® Red reagent and horseradish peroxidase. H_2O_2 standard curve was drawn. Standard curve was prepared via diluting the 20 mM H_2O_2 working solution into 1X Reaction buffer to produce H_2O_2 concentrations of 0 to 10 μ M. The Amplex Red Reagent working reaction mixture was also added. Fluorescence was measured using the multimode microplate reader Biotek Synergy (VT, USA), which has an excitation wavelength of 530 nm and a detection wavelength of 590 nm.

Statistical analysis

After data collection, SPSS software was used for analysis, and the results were given as mean \pm standard error of mean. The experiment groups' findings were compared using the Paired Samples t-test. ** Denoted $p < 0.01$, and *** denoted $p < 0.001$ were utilized as significant indications.

Results

Time-dependent H_2O_2 production in control C6 cells and after 3BP treatment

30 minutes after adding the new DMEM medium, the relative fluorescence units (RFU) was detected in the control wells to be 9871.5 ± 546.54 RFU, while 3BP-treated C6 cells significantly gave 13643.83 ± 149.87 RFU ($p < 0.001$). 45 minutes after adding the new DMEM medium, the relative fluorescence units (RFU) was detected in the control wells to be 9060.12 ± 465.94 , while 3BP-treated C6 cells significantly gave 12396.66 ± 96.67 RFU ($p < 0.001$). 60

minutes after adding the new DMEM medium, the relative fluorescence units (RFU) was detected in the control wells to be 3348.08 ± 114.79 , while 3BP-treated C6 cells significantly gave 11015.72 ± 46.6 RFU ($p < 0.001$). 120 minutes after adding the new DMEM medium, the relative fluorescence units (RFU) was detected in the control wells to be 1815 ± 48.4 , while 3BP-treated C6 cells significantly gave 12329 ± 630.15 ($p < 0.001$), (Fig.2A-D).

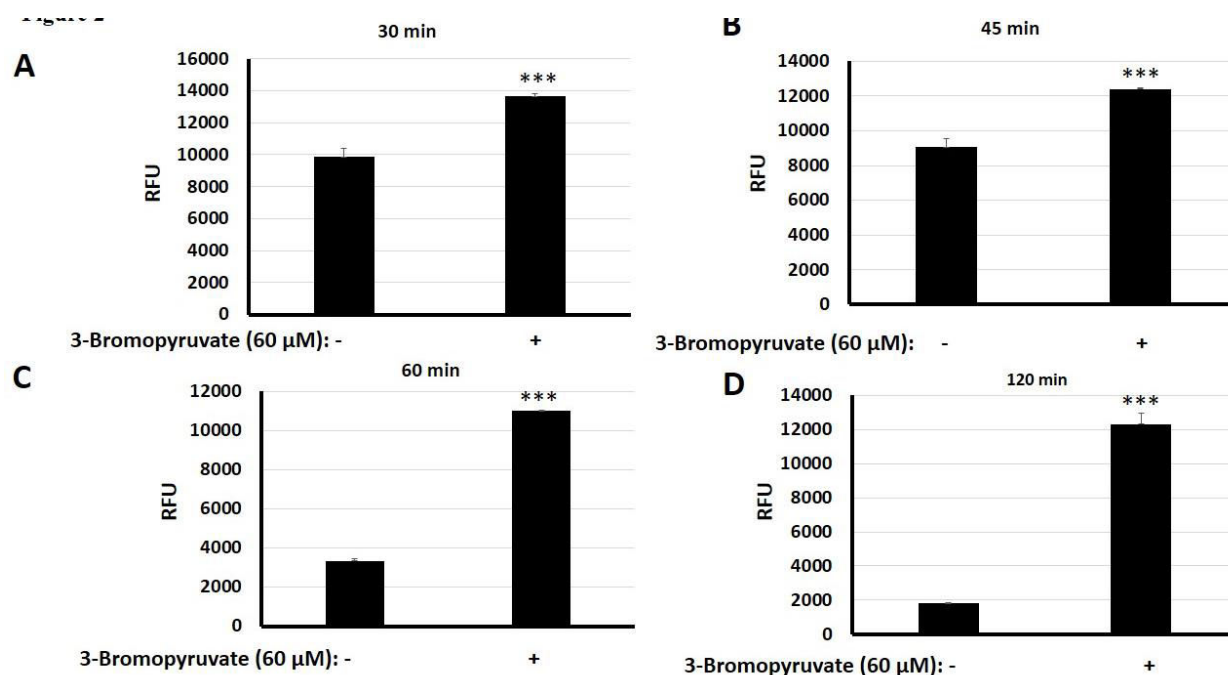


Fig. 2. 3-Bromopyruvate induced the formation of H_2O_2 significantly (measured in RFU) in cultured C6 glioma cells compared to the untreated control in a time-dependent manner. A. 30 minutes after adding the fresh nutrient DMEM medium. **B.** 45 minutes after adding the fresh nutrient DMEM medium. **C.** 60 minutes after adding the fresh nutrient DMEM medium. **D.** 120 minutes after adding the fresh nutrient DMEM medium.

Time-dependent H_2O_2 production in C6 cells upon adding serial doses of pyruvate and lactate

The old DMEM medium was removed and cultured C6 cells were washed and received fresh medium containing serial doses of exogenous pyruvate (1, 10, 50 and 100 mM). Other cultured C6 cells received serial doses of exogenous lactate (1, 10, 50 and 100 mM). Cellular production of H_2O_2

was measured using Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Molecular probes). H_2O_2 produced was measured in RFU. 30 minutes after adding serial doses of lactate (1, 10, 50 and 100 mM) gave the following RFU readings (Mean \pm SEM): 13079.87 ± 961.93 , 11993 ± 675.17 , 11100.5 ± 515.48 , and 10633.25 ± 516.27 , respectively. 30 minutes after adding serial doses of pyruvate (1, 10,

50 and 100 mM) gave the following RFU readings (Mean± SEM): 3581.75± 88.27, 2885.25± 70.04, 1587.75± 54.09, and 1163± 31.22, respectively (**Fig.3A**).

45 minutes after adding serial doses of exogenous lactate (1, 10, 50 and 100 mM) gave the following RFU readings (Mean± SEM): 11467.12± 775.98, 10587.37± 565.13, 9945.25± 398.71, and 9546.87± 424.32, respectively. 45 minutes after adding serial doses of pyruvate (1, 10, 50 and 100 mM) gave the following RFU readings (Mean± SEM): 3527.5±73.98, 2883.25± 52.68, 1614.75± 45.11, and 1184.12±27.33, respectively, (**Fig.3B**)

60 minutes after adding serial doses of exogenous lactate (1, 10, 50 and 100 mM) gave the following RFU readings (Mean± SEM): 3716.5±612.42, 3911.875±612.1,

7624.25±605.23, and 9716.5±925.65, respectively. 60 minutes after adding serial doses of pyruvate (1, 10, 50 and 100 mM) gave the following RFU readings (Mean± SEM): 1994.37±389.74, 1733.75±342.93, 1156.87±178.7, and 1129.12±86.76, respectively, (**Fig.3C**).

120 minutes after adding serial doses of lactate (1, 10, 50 and 100 mM) gave the following RFU readings (Mean± SEM): 2061.75±62.73, 2176.5±46.43, 3929.75±304.24, and 4823.25±51.4, respectively. 120 minutes after adding serial doses of pyruvate (1, 10, 50 and 100 mM) gave the following RFU readings (Mean± SEM): 1001.25±38.62, 906.25±68.7, 709.25±127.16, and 859.5±18.99, respectively (**Fig.3D**).

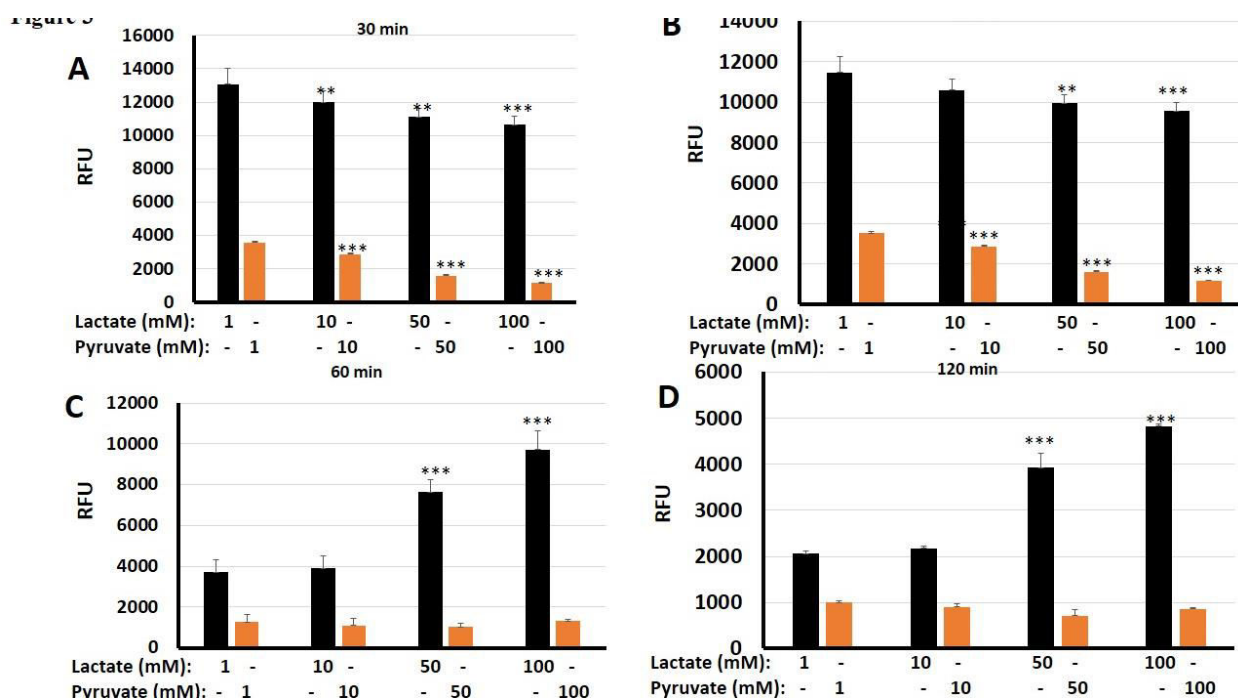


Fig. 3. Pyruvate significantly scavenged the formed H_2O_2 in cultured C6 glioma cells in a time-dependent manner while lactate does not. A. 30 minutes after adding the fresh nutrient DMEM medium. Although higher doses of lactate were associated with H_2O_2 , that was still higher than the baseline values. **B.** 45 minutes after adding the fresh nutrient DMEM medium. Although higher doses of lactate were associated with H_2O_2 , that was still higher than the baseline values. **C.** 60 minutes after adding the fresh nutrient DMEM medium. **D.** 120 minutes after adding the fresh nutrient DMEM medium.

Discussion

In cancer cells, intrinsic oxidative stress increases the production of reactive oxygen species in prostate and other cancers and may contribute to cancer progression due to its stimulating effect on cancer growth. Mitochondrial DNA damage was reported to be sensitive to exogenous H_2O_2 but independent of cellular ROS production in prostate cancer cells. Exogenous H_2O_2 induces preferential cytotoxicity in aggressive prostate cancer than normal cells (Chan et al., 2011). It was reported that Mitochondrial $O_2^{\cdot-}$ and H_2O_2 mediate glucose deprivation-induced oxidative stress in human cancer cells (Ahmad et al., 2005). Chronic oxidative stress increases the growth and tumorigenic potential of cancer cells (Mahalingaiah and Singh, 2014). Such oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells (Chen et al., 2008). The majority of brain tumors overexpress manganese superoxide dismutase (MnSOD), an enzyme that converts superoxide anion into H_2O_2 . The high expression level of MnSOD is linked to tumors' poor prognosis. By activating AKTs and ERKs, H_2O_2 would aid in the migration and invasion of glioma cells that is facilitated by MnSOD. It was shown that over-expression of MnSOD enhanced glioma migration/invasion and raised the level of H_2O_2 . The activation of phosphatidylinositol-3-kinases (PI3Ks) and mitogen-activated protein kinases (MAPKs), which include AKTs, s6-ribosomal protein, ERKs, and JNKs, was strongly enhanced by increased MnSOD. Increases in the matrix metalloproteinase proteins (MMPs) as MMP-9 and MMP-1 were also linked to over-expression of MnSOD. The general reactive oxygen species scavenger N-acetyl-l-cysteine (NAC), over-expression of the H_2O_2 -detoxifying enzyme mitochondrial catalase, and particular inhibitors of AKTs

or ERKs inhibited the promotion of migration/invasion, activation of PI3Ks and MAPKs, and up-regulation of MMPs (Li et al., 2011).

In our earlier studies and published data, the author and Japanese co-researchers confirmed that the promising anticancer drug 3BP is a complete biochemical agent to the tumor's product lactate (a pro-oxidant) and its analog pyruvate (an antioxidant). 3BP induced the production of significant amounts of H_2O_2 and that was scavenged by its analog pyruvate but not by the other analog lactate (figure 1) (El Sayed et al., 2012c). The author then suggested 3BP to be metabolized via glutathione conjugation to delineate the potential of -SH (sulfhydryl)-containing antioxidants to inhibit 3BP-induced effects and the implications of serum and tissue glutathione on 3BP bioavailability and pharmacological actions (El Sayed et al., 2017).

In this in vitro experimental study, C6 glioma cells induced the formation of a significant quantity of H_2O_2 (measured in RFU) at baseline (30 minutes after replacing the old culture medium and adding the fresh nutrient medium) that significantly increased upon adding 3BP treatment ($p < 0.001$). This partly explains 3BP-induced glioma cell death to occur via an oxidative stress mechanism (Fig.2A) and the author termed that oxidative stress-energy depletion therapy in his earlier studies (El Sayed et al., 2012b). The baseline H_2O_2 levels started to decrease in the freshly added culture medium possibly due to a scavenging effect induced by the medium's antioxidants. However, this did not apply to 3BP-induced H_2O_2 formation which significantly persisted ($p < 0.001$) and did not decrease in subsequent time points (Fig. 2A-D). Likewise, 45 minutes later, the baseline H_2O_2 levels in the newly supplied culture media dropped, presumably as a result of the antioxidant constituents in the medium that

scavenged hydrogen peroxide. Noteworthy, 3BP-induced H_2O_2 production was not affected and continued to persist considerably ($p < 0.001$) (**Fig.2B**). Interestingly, baseline H_2O_2 produced in the freshly added culture medium persisted to decrease after one hour (Figure 2C) and after 2 hours (**Fig.2D**) while 3BP-induced H_2O_2 production was significantly high at all the same previously mentioned levels and did not decrease.

Then the author investigated the time-dependent effects of adding serial doses of exogenous pyruvate (in millimolar range) and exogenous lactate (in the same millimolar range) on C6 glioma cells-induced H_2O_2 production in the freshly added culture medium compared at the same different time points. 30 minutes after adding the new culture medium to C6 glioma cells, H_2O_2 induced by the cancer cells was still high (more than the baseline values in **Fig. 2A-D**) upon adding serial doses of lactate. This study's data reported a significant decrease of RFU corresponding to the increased doses of added lactate (**Fig.3A**) but the values were still more than the baseline values i.e. exogenous lactate did not scavenge H_2O_2 . This is in exact agreement with the previous data published by the author and Japanese co-researchers where lactate did not scavenge either endogenously produced H_2O_2 or exogenously added H_2O_2 (**El Sayed et al., 2012c**). Despite the added large doses of lactate, RFU was still above the baseline values, which suggests that lactate has no scavenging effect against H_2O_2 . The decrease in RFU induced by higher doses of lactate (at 50 mM and 100 mM) may possibly be due to the acute effects of exogenously added lactate on C6 glioma cells that needs further research investigations.

Interestingly, serial doses of exogenously added pyruvate (1, 10, 50 and 100 mM) significantly and maximally scavenged H_2O_2

produced by the cultured C6 glioma cells at all the investigated doses and at all the measured time points ($p < 0.001$) (**Fig.3A**). Such pyruvate-induced decrease in H_2O_2 (detected by RFU) was far below the baseline values detected in **Fig. 2A-D**. Likewise, a similar picture was observed 45 minutes after adding serial doses of lactate and pyruvate to the freshly added DMEM culture medium ($p < 0.001$) (**Fig.3B**). Interestingly, when lactate was added in successive doses to C6 glioma cells 60 minutes after the new DMEM culture medium was added, the H_2O_2 produced by the cancer cells remained high (above the baseline values in **Fig.2A-D**). Exogenous lactate did not scavenge H_2O_2 , according to the study's data, which showed a considerable increase in RFU in response to increasing the doses of added lactate (**Fig.3C**). In addition, the values were still higher than baseline. The opposite occurred with pyruvate where there was a time-dependent decrease in RFU and that was more with serial doses of added pyruvate (a scavenging effect to H_2O_2) (**Fig. 3A-D**).

The H_2O_2 generated by the cancer cells remained high (above the baseline values seen in **Fig. 2A-D**) even after lactate was introduced to C6 glioma cells in successive doses 120 minutes after the addition of the new DMEM growth medium. In this study's data, there was a significant rise in RFU in response to increasing the doses of added lactate, which indicated that exogenous lactate did not scavenge H_2O_2 (**Fig.3D**). Furthermore, the readings remained above the baseline. Compared to the values gained after 30 minutes, 45 minutes and after 60 minutes, the RFU decreased more after 120 minutes in a time-dependent manner, but it decreased more with each additional pyruvate dosage (which has the scavenging action of H_2O_2) (**Fig. 3A-D**).

The author then reported his expert opinion on the molecular origin of the

Warburg effect (persistent endogenous and continuous lactate production by cancer cells even upon oxygen existence) to result from a closed cycle and vicious circle between the two reversible glycolytic enzymes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH) where the two biochemical reactions catalyzed by both turn to be irreversible resulting in favoring lactate production but not pyruvate (El Sayed, 2023).

Conclusion

C6 glioma cells induce the formation of H₂O₂ at baseline and this decreased with adding the new culture medium at different sequential time points. 3BP induced the generation of H₂O₂ that persisted for at least two hours. Serial doses of lactate did not scavenge C6 glioma-induced H₂O₂ production. Serial doses of pyruvate significantly and maximally scavenged C6 glioma-induced H₂O₂ production.

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Conflict of interest

The author declares that there is no conflict of interest

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