



Effect of hypoxia on purine metabolism in human skeletal muscle cells

Efecto de la hipoxia en el metabolismo de purinas en células de músculo esquelético de humanos

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ABSTRACT

Mammals experience some degree of hypoxia during their lifetime. In response to hypoxic challenge, mammalian cells orchestrate specific responses at transcriptional and posttranslational level, which lead to changes in purine metabolites in order to cope with threatening conditions. The aim of this study was to evaluate the response of the enzymes involved in purine metabolism at human muscle cells under hypoxic conditions. Muscle cells in culture were exposed to hypoxia and the enzymatic activity of inosine monophosphate dehydrogenase (IMPDH), xanthine oxidase (XO), purine nucleoside phosphorylase (PNP) and hypoxanthine guanine phosphoribosyl transferase (HGPRT) as well as their transcript expression were quantified under normoxic and hypoxic conditions. Purine metabolite (hypoxanthine (HX), xanthine (X), uric acid (UA), inosine monophosphate (IMP), inosine, nicotinamide adenine dinucleotide (NAD⁺), adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), guanosine diphosphate (GDP) and guanosine triphosphate (GTP)) concentrations were also quantified. Significant reduction of IMPDH activity as well as in HX and IMP concentrations ($p < 0.05$) were observed after hypoxia, suggesting a decrease in the purines *de novo* synthesis. After hypoxia a global reduction of transcripts was observed, suggesting a reduction of the metabolic machinery of purine metabolism to new steady states that balance ATP demand and ATP supply pathways.

Key words: hypoxia, cell culture, purines, salvage pathway.

RESUMEN

Los mamíferos experimentan cierto grado de hipoxia durante su vida. Como respuesta al reto de hipoxia, las células de mamíferos orquestan respuestas específicas a nivel transcripcional y postraduccionales que conducen a cambios en los metabolitos de purinas para hacer frente a las condiciones amenazantes. El objetivo de este estudio fue evaluar la respuesta de las enzimas involucradas en el metabolismo de las purinas de células musculares humanas a condiciones hipóxicas. Las células musculares en cultivo se expusieron a hipoxia y la actividad enzimática de la inosina monofosfato

deshidrogenasa (IMPDH), la xantina oxidasa (XO), la purina nucleósido fosforilasa (PNP) y la hipoxantina guanina fosforribosil transferasa (HGPRT), así como su expresión de transcripción, se cuantificaron bajo condiciones de normoxia e hipoxia. Los metabolitos de purina (hipoxantina (HX), xantina (X), ácido úrico (UA), monofosfato de inosina (IMP), inosina, dinucleótido de nicotinamida y adenina (NAD⁺), adenosina, monofosfato de adenosina (AMP), difosfato de adenosina (ADP), trifosfato de adenosina (ATP), difosfato de guanosina (GDP) y trifosfato de guanosina (GTP)) también se cuantificaron. Se observó una reducción significativa de la actividad de IMPDH y de las concentraciones de HX e IMP ($p < 0.05$) después de la hipoxia, lo que sugiere una disminución de la síntesis *de novo* de purinas. Después de la hipoxia, se observó una reducción global de la expresión transcripcional, lo que sugiere una reducción de la maquinaria metabólica del metabolismo de las purinas a nuevos estados estacionarios que equilibran la demanda de ATP y las vías de suministro de ATP. **Palabras clave:** hipoxia, cultivo celular, purinas, vía de rescate.

INTRODUCTION

Purine metabolism constitutes a key pathway for every organism. It is involved in DNA and RNA synthesis and degradation, and involves the production of ubiquitous metabolites, such as adenosine triphosphate (ATP) (Yin *et al.*, 2018). There are two main routes for the synthesis of nucleotides, the *de novo* biosynthetic pathway and the complementary salvage pathway. The *de novo* biosynthetic pathway is responsible for the production of inosine monophosphate (IMP) in a twelve-step biosynthetic process with consumption of ATP (Pang *et al.*, 2012; Yin *et al.*, 2018). The complementary salvage pathway, however, converts extracellular nucleobases or degraded purine metabolites into the corresponding nucleosides and nucleotides (Nelson *et al.*, 2008), and obviates energetically expensive *de novo* synthesis of these compounds.

When mammals are exposed to oxygen limiting situations, such as breath-hold diving induced hypoxia or ische-

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nia, systemic and intracellular changes operate together to minimize injury and restore adequate oxygenation. During hypoxia, mammalian tissues cannot produce enough energy to maintain essential cellular processes (Giordano, 2005); thus, a reduction of ATP synthesis is produced, promoting the degradation of purine nucleotides (Nelson *et al.*, 2008) which are accumulated in the cells (Raivio *et al.*, 2001). The catabolic compounds derived from ATP breakdown, hypoxanthine (HX), inosine (I), xanthine (X), uric acid (UA) and adenosine (A), have been widely used as markers of tissue hypoxia (Maiuolo *et al.*, 2016).

Purine degradation involves the activation of purine nucleotide phosphorylase (PNP, E.C. 2.4.2.1) and xanthine oxidase (XO, E.C. 1.1.3.22) (Maiuolo *et al.*, 2016). PNP catalyzes the reversible conversion of nucleosides (guanosine, xanthosine, inosine) to purine bases (guanine, xanthine, and hypoxanthine) (Dudzinska *et al.*, 2006), while XO catalyzes the oxidation of hypoxanthine and xanthine to uric acid (Vorbach *et al.*, 2003). Salvage of purines occurs through two main enzymes, adenine phosphoribosyltransferase (APRT), which mediates the transfer of phosphoribosyl-1-pyrophosphate (PRPP), a high energy sugar phosphate, to adenine, to form adenosine monophosphate (AMP), and hypoxanthine-guanine phosphoribosyltransferase (HGPRT, E.C. 2.4.2.8), which forms IMP and guanosine monophosphate (GMP) (Nelson *et al.*, 2008; Zhang *et al.*, 2008). The enzyme inosine 5-monophosphate dehydrogenase (IMPDH, E.C. 1.1.1.205) uses IMP to produce xanthosine 5-monophosphate (XMP) and, ultimately, ATP (Cherin *et al.*, 2006).

Oxygen limitation has a tissue-dependent effect (Wagner, 2008), which triggers transcriptional (Huang *et al.*, 2004; Rocha, 2007) and posttranslational (Schumacker, 2011) modifications to the enzymes involved in the nucleotide synthesis and degradation. The *de novo* biosynthetic pathway is energy demanding, requiring five ATP molecules, two glutamine and formate molecules, and one glycine, aspartate and carbon dioxide molecules to generate one IMP molecule. During 24h hypoxia in human cells, a significant increase in hypoxanthine (HX) level is produced (Nagao *et al.*, 2018), which is used by XO. Hypoxia, as well as other stimuli, including growth factors, cytokines and hormones, upregulates XO mRNA expression (Battelli *et al.*, 2016). However, XO activity increases in response to hypoxia without changes in mRNA or protein levels (Berry and Hare, 2004), indicating that XO is modulated at a posttranslational level, mainly through phosphorylation (Kayyali *et al.*, 2001).

Purine recycling in mammals increases in response to prolonged fasting and ischemia (López-Cruz *et al.*, 2014). PNP activity increases significantly during ischemia (Vannoni *et al.*, 2004) and its enzymatic deficiency in purine metabolism leads to several diseases, including Lesch-Nyhan syndrome, clinical gout, nephropathy and neurological disease (Nyhan, 2005). HPGRT activity in mammals plasma indicates an elevated purine recycling rate to avoid accumulation of non-recyclable purines (xanthine and uric acid) (López-Cruz *et al.*, 2014), which rise the need for a comprehensive unders-

tanding of the regulation of enzymes involved in the purine metabolism.

The aim of this study was to analyze and compare the enzymatic activity and transcript expression of the proteins involved in purine degradation (XO, PNP) and salvage pathway (HPGRT), and the concentration of metabolites involved in purine metabolism after hypoxic conditions in human muscle cells.

MATERIAL AND METHODS

Cell culture

Human skeletal muscle cells (36 cell culture boxes) were cultured in DMEM/F12 50 (Cellgro) supplemented with 15% fetal bovine serum (FBS, Gibco), antibiotics (50 U mL⁻¹ penicillin, 50 µg mL⁻¹ streptomycin), 2 mM glutamax (Gibco), 25 mM HEPES (pH 7.4) and 100 µM sodium pyruvate (Gibco). Cells (18 culture boxes) were cultured in a humid atmosphere at 35°C and serially passaged upon 80% confluence. Hypoxic conditions were obtained by placing the cells (18 cell culture boxes) in a sealed hypoxic workstation for 1 h, medium degas time took around 5 minutes. Afterwards, control cells (normoxia) and hypoxic cells (hypoxia) with 80% confluency were detached from the box using trypsin 0.25% (GIBCO) by a 4 min incubation at 35°C, then trypsin was inactivated by the addition of fresh medium. Cell suspension was recovered in 15 mL tubes, centrifuged at 1200 rpm for 10 min. Supernatant was discarded and the pellet (cells) was re-suspended in 500 µL of medium containing 1% DMSO for metabolite and protein analysis, and in tubes containing TRIzol® Reagent (15596, Invitrogen, Carlsbad, CA, USA) for gene expression. This process was performed for nine cell culture boxes for each treatment. All samples were stored at -80°C until further analysis.

Protein extraction and quantification

Prior to analysis, cells (nine cell culture boxes per treatment) in medium containing DMSO were centrifuged at 1200 rpm for 15 min to remove the medium. Afterwards, 350 µL of deionized water were added and then vortexed for 30 s, followed by three cycles of freezing/thawing for cellular disruption. Finally, samples were centrifuged at 17900 x g for 15 min at 4°C. Supernatant, containing soluble proteins was recovered and stored at -80°C until further analysis. Total soluble protein was quantified using a commercial kit (Bio-Rad Laboratories, Inc. Hercules, CA, USA) based on the method described by Bradford (1976). Bovine serum albumin (BSA) was used as standard, and results expressed in mg mL⁻¹.

Purine metabolite concentration

Concentration of purines (HX, X, A, I, NAD⁺, UA, IMP, AMP, ADP, ATP, XMP, GDP and GTP) was quantified in human muscle cell culture samples from normoxia and hypoxia experiments by high performance liquid chromatography (HPLC) (Waters 2695, Milford, MA, USA) (Gianattasio *et al.*, 2003). Samples were treated with 0.5 M cold perchloric acid to precipitate soluble protein, and the solution was

then neutralized with 0.5 M potassium hydroxide. The potassium perchlorate produced from the neutralization was eliminated by centrifugation at $17900 \times g$ for 10 min at 4°C . The supernatant containing the purine metabolites was recovered and filtered through a Nylon membrane filter of $0.22 \mu\text{m}$ (Int. Millipore, Bedford, MA, USA) into a HPLC vial for analysis, the medium was also analyzed to evaluate if the metabolites were expelled from the cell during hypoxia. Purine metabolites were analyzed by HPLC in an analytical column Supelcosil C-18 ($150 \times 4.6 \text{ mm ID}$, $3 \mu\text{m}$ particle size; Waters). Metabolites were separated using a gradient from 0 to 19 min, beginning at 100% of buffer A (100 mM KH_2PO_4 , 8 mM TBA pH 6.0) to 50% buffer B (100 mM KH_2PO_4 , 8 mM TBA, 30% acetonitrile pH 6.0) at a flow rate of 1.3 mL min^{-1} . The eluate was monitored at 214 nm. The concentration of each purine metabolite was quantified using standard curves of a purine standards mixture (0.625 to $100 \mu\text{M}$), expressing the metabolite concentrations as $\mu\text{M mg}^{-1} \text{ protein}$.

Enzymatic assays

Inosine 5'-monophosphate dehydrogenase (IMPDH, E.C. 1.1.1.205) activity

IMPDH activity was measured by quantifying the concentration of xanthosine 5'-monophosphate (XMP), resulting from the conversion of IMP, using HPLC (Waters 2695, Mildford, MA, USA) (Glander *et al.*, 2001). Enzymatic assays were performed in 40 mM sodium phosphate buffer pH 7.4, containing 0.5 mM IMP, 0.25 mM NAD^+ and 50 mM KCl. Reactions were incubated for 3 h at 37°C , subsequently 4 M cold perchloric acid was added to stop the reaction; the reaction mixture was neutralized with 5 M potassium carbonate and finally centrifuged at $15800 \times g$ for 5 min, the supernatant was recovered and filtered through a nylon membrane filter of $0.22 \mu\text{m}$ (Int. Millipore, Bedford, MA, USA) into an HPLC vial for analysis. XMP was analyzed by HPLC on an analytical column ODS hypersyl 125 AQ ($250 \times 3.1 \text{ mm ID}$, $3 \mu\text{m}$ particle size; Waters). XMP was separated using a gradient from 2 to 20 min, beginning at 100% of buffer A (100 mM KH_2PO_4 , 8 mM TBA pH 6.0) to 60% buffer B (100 mM KH_2PO_4 , 8 mM TBA, 30% acetonitrile pH 6.0) at flow rate of 1.1 mL min^{-1} . The eluate was monitored at 214 nm. The XMP concentration was quantified using XMP standard curves (1.5 to $50 \mu\text{M}$). One unit of IMPDH is defined as the amount of enzyme needed to produce 1 mM of XMP, and the activity expressed as $\mu\text{M mg}^{-1} \text{ protein h}^{-1}$.

Purine nucleotide phosphorylase (PNP, E.C. 2.4.2.1) activity

PNP activity was measured indirectly by the oxidation of 3, 5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone to N-(4-antipiryl)-3-chloro-5-sulphonate-b-benzoquinonamionio, by the hydrogen peroxide formed in the PNP-xanthine oxidase reaction (Chu *et al.*, 1989). Briefly, the enzymatic reaction was performed in 22 mM potassium phosphate buffer pH 7.5, containing 25 U XO, 2000 U of horseradish peroxidase (HRP), 160 mM 4-aminoantipyrine, 120 μM

potassium ferrocyanide, 8 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, and 12 mM inosine. Production of N-(4-antipiryl)-3-chloro-5-sulfonate-p-benzoquinone-monoimine was recorded at 520 nm in a spectrophotometer (Beckman Coulter DU 800, Fullerton, CA, USA) and the change of absorbance was recorded every 5 s for 3 min. The activity of PNP expressed as units (U) $\text{mg}^{-1} \text{ protein}$, defining a unit of PNP activity as the amount of enzyme necessary to deplete $1 \mu\text{M}$ of inosine per min at 25°C .

Hypoxanthine-guanine phosphoribosyltransferase (HGPRT, E.C. 2.4.2.8) activity

HGPRT activity was measured by quantifying the rate of IMP production, which is oxidized by IMPDH, using the PIERCE HPRT Assay Kit (NOVOCIB, Lyon, France) following the manufacturer's instructions. Briefly, enzymatic assay was performed in reaction buffer (1X) containing IMPDH, DTT, NAD^+ and PRPP. The reaction mixture was recorded every 5 min for 120 min at 340 nm using a microplate reader (Multiskan FC, Thermo Scientific, Finland), and HGPRT activity expressed as $\text{nmol mg}^{-1} \text{ protein h}^{-1}$.

Xanthine oxidase (XO, E.C. 1.17.3.2) activity

XO activity was measured using an Amplex[®] Red Xanthine/Xanthine oxidase assay kit (A22182; Invitrogen, Paisley, UK). The quantification is based on the superoxide anions ($\text{O}_2^{\cdot-}$) produced by XO, which are degraded to hydrogen peroxide (H_2O_2), which reacts stoichiometrically with Amplex[®] Red reagent to generate the oxidation product, resofurin. Briefly, the enzymatic assay was performed in reaction buffer (1X) containing $100 \mu\text{mol L}^{-1}$ Amplex Red reagent solution, 0.4 U mL^{-1} HRP and $200 \mu\text{mol L}^{-1}$ hypoxanthine, incubated for 30 min at 37°C . The presence of resofurin was recorded at 550 nm using a microplate reader (Multiskan FC, Thermo Scientific, Finland), and the XO activity expressed as mU mg^{-1} . One unit of XO activity is defined as the amount of enzyme required to produce $1 \mu\text{M}$ of UA from HX per min at 25°C .

Quantification of XO, PNP, IMPDH and HGPRT transcripts RNA extraction and cDNA synthesis

Total RNA was isolated from 1×10^6 cells of skeletal muscle cells from human cell cultures (normoxia and hypoxia) using TRIzol[®] Reagent (15596, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Contaminating genomic DNA was removed using DNase I (AMPD-1, Sigma-Aldrich Chemical), and total RNA concentration was spectrophotometrically determined at 260 nm. RNAs ($1 \mu\text{g}$) were reverse transcribed into cDNA using the QuantiTect[®] Reverse Transcription kit (205313, Qiagen) using random hexamer primers and according to the manufacturer instructions. cDNA samples were stored at -80°C until further analysis.

Real-time PCR analysis

Determination of relative mRNA expression of IMPDH, XO, HGPRT and PNP was performed by real-time PCR, using

the ABI 7000 Real Time PCR (Applied Biosystems). cDNA was diluted (1:5) and analyzed using the QuantiTect[®] SYBR[®] Green PCR Kit (204145, Qiagen). The reaction mixture contained 10 μL of SYBR[®] Green, 1.0 μL of forward and reverse primers (10 μM each), 5.0 μL of cDNA (50 ng), and 4.0 μL of sterile, distilled water. Controls without template were included to ensure absence of contaminating DNA. PCR reactions were carried out as follows, an initial cycle at 95°C for 15 min to activate the HotStarTaq[®] DNA polymerase, followed by the denaturalization of the cDNA at 94°C for 15 s, 40 cycles at 94°C for 15 s each, 60°C for 30 s, and 72°C for 30 s. The specificity of each primer set was validated by the construction of a melting curve, constructed after PCR by increasing the temperature from 75°C to 95°C at a rate of 0.3°C every 20 s. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was determined as housekeeping gene. The relative expression of target mRNA was normalized to the amount of GAPDH in the same cDNA using the standard curve method. Amplification efficiency for each analyzed gene was ~98%, using dilution from $0.25 \times 10^{-2} \text{ ng } \mu\text{L}^{-1}$ to $0.25 \times 10^{-6} \text{ ng } \mu\text{L}^{-1}$ of PCR fragments. Each RT-PCR data point is average of three independent biological replicates. Primer sequences and accession numbers for the housekeeping gene GAPDH and for the different enzyme genes are included in Table 1.

Ethical aspects

Muscle samples were obtained in accordance with institutional ethical standards and with the 1964 Helsinki declaration and its later amendments from healthy volunteers subjected to programmed cesarean section at the Hospital de Especialidades Médicas Fidepaz in La Paz, Baja California Sur, México. All participants in the study signed the informed consent form, which, together with the research project, were approved by the Baja California Sur Chapter of the National Mexican Academy of Bioethics and registered with the Comité de Ética en Investigación (CONBIOÉTI-CA-09-CEI-009-20160601).

Statistical analyses

Data were tested for normality and homoscedasticity using Kolmogorov-Smirnov ($p < 0.05$) and Levene ($p < 0.05$) tests, respectively. Significant differences in the non-parametric data were determined with Kruskal-Wallis and Mann-Whitney U tests (Zar, 2009). Statistical significance was considered when $p \leq 0.05$, determined using GraphPad Prism version 3.03.

Table 1. Primer sequences for real-time PCR.

Tabla 1. Secuencias de oligonucleótidos para PCR en tiempo real.

Target gene	Sense-primer (5'-3')	Antisense-primer (5'-3')	GenBank accession no.	Size (bp)
IMPDH	GGCTCCATCTGCATCACCA	CCATGCCCCGGTACTTCTTG	NM_000883	262
HPRT	GACTGAAGAGCTACTGTAATG	CACCAGCAAGCTTGAACC	NM_000194	198
PNP	CTGTGTGATGATGCAGGGCAG	TCATTGGGCCCTCTGAGAGG	NM_000270	225
XO	ACAGAACACCATGAAGACCC	CTGCCACCAGTTATCAGCATG	NM_000379	224
GADPH	GGAAACTGTGGCGTGATGG	CCTGCTTACCACCTTCTTG	AF261085	216

RESULTS

Enzyme activity

Figure 1 show the activity of IMPDH, PNP, XO and HGPRT from human skeletal muscle cells after normoxia and hypoxia conditions. IMPDH activity was significantly higher in normoxic ($46.59 \pm 11.95 \mu\text{M mg}^{-1} \text{ protein h}^{-1}$) than hypoxic cells ($22.09 \pm 2.74 \mu\text{M mg}^{-1} \text{ protein h}^{-1}$) ($p < 0.036$). There were no significant differences in PNP, XO and HGPRT activities between treatments ($p > 0.05$).

Purine metabolite concentration

The concentration of purine bases (HX, X), nucleosides (I, A), nucleotides (AMP, GDP, ADP, GTP, ATP), NAD⁺ and UA were measured by HPLC after normoxia and hypoxia treatments in human skeletal muscle cells (Table 2). UA was not detected in neither normoxic nor hypoxic cells. There

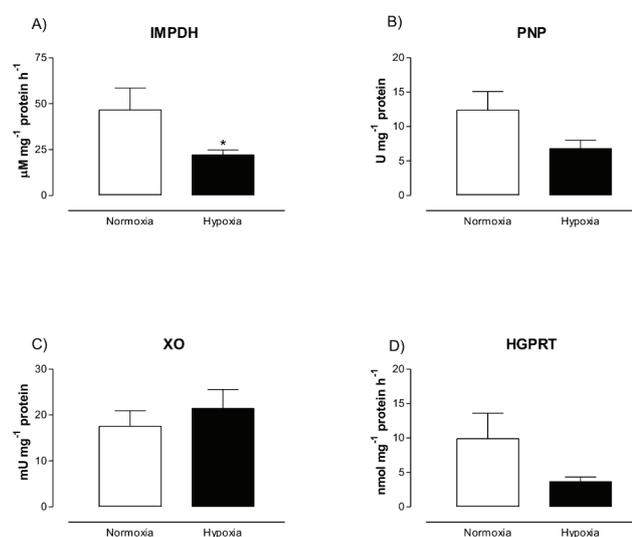


Figure 1. Effect of hypoxia on enzymes involved in purine metabolism.

A) Inosine 5'-monophosphate dehydrogenase (IMPDH) activity, B) purine nucleotide phosphorylase (PNP) activity, C) xanthine oxidase (XO) activity and D) hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity. Skeletal muscle cells were incubated under normoxic or hypoxic conditions for 1 h and cells were collected and tested for each enzymatic activity. Data are shown as mean \pm S.E. of at least five independent biological experiments by triplicate. * denotes differences between treatments ($p < 0.05$).

Figura 1. Efecto de la hipoxia en las enzimas involucradas en el metabolismo de purinas. A) Actividad de inosina monofosfato dehidrogenasa (IMPDH), B) Actividad de purina nucleótido fosforilasa (PNP), C) Actividad de Xantina oxidasa y D) hipoxantina guanina fosforibosil transferasa (HPGRT). Las células de músculo esquelético fueron incubadas bajo condiciones de normoxia o hipoxia por 1 h y las células fueron colectadas y evaluadas para cada ensayo enzimático. Los datos representan medias \pm D.S. de al menos cinco experimentos biológicos independientes realizados por triplicado. Asteriscos (*) denotan diferencias estadísticas entre tratamientos ($p < 0.05$).

Table 2. Purine metabolite concentrations in human skeletal muscle cells exposed to hypoxia.

Tabla 2. Concentración de metabolitos de purina en células de músculo esquelético de humano expuestos a hipoxia.

Metabolite	Normoxia	Hypoxia
HX	1.54 (0.81, 2.17)	0.76 (0.62, 0.99)*
Xanthine	2.04 (0.99, 3.35)	1.52 (0.98, 2.06)
UA	ND	ND
IMP	1.04 (0.55, 2.12)	0.42 (0.29, 0.62)*
Inosine	4.71 (2.5, 7.22)	3.33 (1.86, 4.38)
NAD ⁺	0.25 (0.08, 0.50)	0.21 (0.09, 0.78)
Adenosine	0.18 (0.02, 8.34)	1.11 (1.00, 1.15)
AMP	0.18 (0.02, 8.34)	1.11 (1.10, 1.15)
GDP	0.07 (0.03, 0.11)	0.05 (0.02, 0.08)
ADP	0.37 (0.06, 7.17)	0.09 (0.03, 2.68)
GTP	4.56 (2.24, 6.88)	0.08 (0.01, 2.20)
ATP	0.31 (0.17, 0.87)	0.16 (0.09, 0.60)

Hypoxanthine (HX), xanthine, uric acid (UA), inosine monophosphate (IMP), inosine, nicotinamide adenine dinucleotide (NAD⁺), adenosine, adenosine monophosphate (AMP), guanosine diphosphate (GDP), adenosine diphosphate (ADP), guanosine triphosphate (GTP) and adenosine triphosphate (ATP). Data are expressed in $\mu\text{M mg}^{-1}$ protein and represent five independent experiments for each treatment. Data are presented as median (25th-75th percentiles). ND = not detected. *=Significant differences between treatments, $p < 0.05$.

was a reduction in the content of most of the metabolites, purine bases, nucleosides and nucleotides, in cells under hypoxic conditions as compared to normoxic cells; however, differences were only statistically significant for HX and IMP concentrations ($p = 0.0031$).

Hypoxia downregulates gene expression of enzymes that participate in purine metabolism

Transcripts encoding for IMPDH, PNP, XO and HGPRT were obtained from human skeletal muscle cells cDNA. The BLAST analysis of the sequences from the obtained fragments (~300 bp), confirmed the identity of the coding sequences obtained which correspond to the enzymes of purine metabolism. Quantitative real-time PCR was used to analyze the levels of expression of mRNAs encoding IMPDH, PNP, XO and HGPRT enzymes involved in purine metabolism (Fig. 2). IMPDH and XO were the most abundant transcripts, showing the highest significant reduction ($p < 0.01$) after hypoxia in contrast to normoxic cells (~99% each). HGPRT mRNA showed a 58% reduction ($p < 0.01$) under hypoxic conditions, while PNP presented the lowest expression from all the transcripts analyzed, and it did not show significant differences in contrast to hypoxic cells.

DISCUSSION

Mammals have developed effective strategies to cope with oxygen limiting situations. To achieve tolerance to acute or chronic hypoxia, the organism reduces metabolism, to prevent cellular injury and maintain functional integrity (Ramirez *et al.*, 2007). Failure of these strategies results in different physiological and pathophysiological conditions

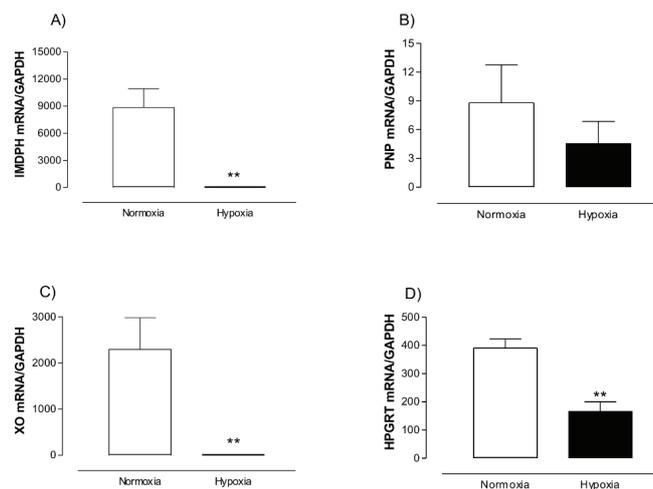


Figure 2. Effect of hypoxia on the expression of the enzymes involved in purine metabolism. A) Inosine 5'-monophosphate dehydrogenase mRNA (IMPDH), B) purine nucleotide phosphorylase mRNA (PNP), C) xanthine oxidase mRNA (XO), and D) hypoxanthine-guanine phosphoribosyltransferase mRNA (HGPRT). Skeletal muscle cells were incubated under normoxic or hypoxic conditions for 1 h, afterwards cells were collected and transcripts were quantified by real-time PCR. Data were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene. Data are shown as mean \pm S.E. of three independent biological experiments by triplicate. *=statistical differences between treatments ($p < 0.05$).

Figura 2. Efecto de la hipoxia en la expresión de las enzimas involucradas en el metabolismo de purinas. A) Actividad de inosina monofosfato dehidrogenasa (IMPDH), B) Actividad de purina nucleótido fosforilasa (PNP), C) Actividad de Xantina oxidasa y D) hipoxantina guanina fosforribosil transferasa (HGPRT) RNAm. Las células de músculo esquelético fueron incubados bajo condiciones de normoxia o hipoxia por 1 h, posteriormente las células fueron colectadas y los transcritos cuantificados por PCR en tiempo real. Los datos fueron normalizados usando gliceraldehído 3-fosfato dehidrogenasa (GAPDH) como gen de referencia. Los datos representan medias \pm D.S. de tres experimentos biológicos independientes realizados por triplicado cada uno. Los asteriscos denotan diferencias estadísticas entre tratamientos ($p < 0.05$).

(e.g. ischemia, inflammation and tumors) (Lahat *et al.*, 2011).

The primary response to hypoxia within the cell ultimately leads to alternative routes of ATP generation (Wheaton and Chandel, 2011). IMPDH is a key enzyme in the purine nucleotide pathway, since it controls the gateway to guanine nucleotides (Hedstrom, 2009). IMPDH activity has been widely studied in several tissues, including plasma, erythrocytes, lymphocytes, spleen and thymus (Jonsson and Carlsten, 2001; Fukuda *et al.*, 2011; Vethe *et al.*, 2014; Citterio-Quentin *et al.*, 2019) from different mammals and aquatic organisms (Velasco-Martinez *et al.*, 2016). Differential activity patterns between tissues have been reported; low activity has been observed in erythrocytes and lymphocytes ($89 \text{ pmol mg Hb}^{-1} \text{ h}^{-1}$, $4514 \text{ pmol mg}^{-1} \text{ protein h}^{-1}$, respectively) compared to human skeletal muscle cells in this study ($46.59 \text{ } \mu\text{M mg}^{-1} \text{ protein h}^{-1}$). Human cells contain two IMPDH genes, which share 84% homology, although protein sequence differences does not affect their kinetic properties (Hedstrom, 2009). In the present study, a partial sequence of IMPDH from human skeletal muscle cells, amplified and sequenced contained a conserved functional region, shared by the two

IMPDH genes. Normoxic cells showed high expression of IMPDH mRNA and IMPDH activity. After hypoxia treatment, IMPDH mRNA and IMPDH activity, as well as the endogenous levels of IMP, were downregulated, suggesting that oxygen limitation transcriptionally affects the *de novo* production of purines, as has been shown in normal lymphocyte cells (Jain *et al.*, 2004) and hypoxia-treated human leukemic cells (Han *et al.*, 2006).

PNP and XO are involved in purine degradation, PNP catalyzes the inosine breakdown to hypoxanthine (Dudzinska *et al.*, 2006), the substrate for xanthine oxidase (Moriwaki *et al.*, 1999). PNP activity has been reported in erythrocytes, liver and plasma of mammals (Schomaker *et al.*, 2013; López-Cruz *et al.*, 2014; Giuliani *et al.*, 2016). Previous reports on PNP activity in human muscle cells show a higher activity in cell cultures than in human muscle homogenates (Jacobs *et al.*, 1988). In this study, muscle cell PNP activity was $12.37 \pm 2.71 \text{ U mg}^{-1}$ under normoxic conditions, while after hypoxia treatment lower PNP activity was observed ($6.79 \pm 1.20 \text{ U mg}^{-1}$), although this decrease was not significant. The same pattern was observed when the PNP mRNA was analyzed. In erythrocytes from rats, changes in PNP activity were evident after seven days of hypoxia (Hosek *et al.*, 1986), while liver from the same species required an hour to stimulate its activity (Rao *et al.*, 1989). In human adipocytes, PNP mRNA expression increased after 24h hypoxia (Nagao *et al.*, 2018). The differential activity and expression pattern of PNP among tissues, might reflect different metabolic demands of each tissue and responses to stress conditions. XO activity in muscle tissue has been reported as less than 0.5 mU g^{-1} tissue (Dorion *et al.*, 1993); in this study, higher levels of XO activity were obtained ($17.55 \pm 3.40 \text{ mU mg}^{-1}$). The hypoxia treatment resulted in a mild increase of XO activity ($21.44 \pm 4.13 \text{ mU mg}^{-1}$); however, this increase was not statistically significant. Previous reports indicate that moderate hypoxia significantly enhances XO activity in endothelial arterial cells (Kelley *et al.*, 2006) and skeletal muscle and human plasma (Terada *et al.*, 1992). The lack of increased activity in this study might be related to the significant reduction of HX levels observed after hypoxia ($1.54 \mu\text{M mg}^{-1}$ under normoxia and $0.76 \mu\text{M mg}^{-1}$ under hypoxic conditions), limiting the catalytic capability of the enzyme. This level is consistent with the absence of UA and low levels of X. Interestingly, XO mRNA expression is modulated by hypoxia in a dose- and time-dependent manner, either by enhancing their expression (moderate hypoxia) or not affecting the gene expression (Poss *et al.*, 1996; Kelley *et al.*, 2006). In this study, XO mRNA in muscle cells was downregulated in hypoxic conditions; these differences might relate to the time of exposure to hypoxia and to the level of regulation that XO presents, which is at transcriptional and posttranslational level (Kayyali *et al.*, 2001; Poss *et al.*, 1996).

The alternate pathway for purine nucleotide formation is the purine salvage pathway, in which instead of generating new purines, preexisting purine bases are taken up for the generation of nucleotides. HGPRT activity in human muscle cells has been previously reported to be $4.8 \pm 0.015 \text{ nmoles mg}^{-1} \text{ h}^{-1}$ (Jacobs *et al.*, 1988), which is in agreement with

the levels obtained in the muscle cell culture under basal conditions ($9.90 \pm 3.6 \text{ nmoles mg}^{-1} \text{ h}^{-1}$) in this study. An increase of HGPRT activity has been observed after prolonged fasting (Soñanez-Organis *et al.*, 2012) and as a compensatory response to hypoxia in mice heart tissue (Wu *et al.*, 2015). Interestingly, cerebral hypoxia-ischemia experiments in rabbits did not show changes in HGPRT activity (Cherin *et al.*, 2006). In this study, HGPRT activity decreased after hypoxia exposure; however, this reduction was not significant at the protein level, but it was significant at the transcriptional level.

The analysis of the purine metabolites showed a significant increase in HX content, which correlates with the low activity of XO observed. These results correlated with previous findings in adipocyte tissues exposed to hypoxia, which showed an increase in HX and acid uric secretion during prolonged hypoxia (Nagao *et al.*, 2018). The significantly reduction of IMP concentration in this study, might be related to an active uptake of this compound by IMPDH, as has been shown in other mammalian tissues, including erythrocytes, placenta, liver and heart (Traut, 1994; Pérez-Milicua *et al.*, 2015).

CONCLUSIONS

The observed reduction in enzyme activities for purine metabolism as well as their transcripts, along with the significant reduction of HX and IMP levels suggest that skeletal muscle cells respond differently to oxygen limitation than other tissues previously studied. This reduction might relate to a decrease of the metabolic machinery to avoid the excessive production of metabolites, which may lead to apoptotic signaling. This could suggest that skeletal muscle cells are able to withstand longer periods of hypoxia, before developing alternative mechanisms to cope with hypoxia effects via the salvage pathway, as has been observed in erythrocytes and plasma. However, further research is necessary to understand the mechanism underlying purine metabolism under hypoxia conditions.

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REFERENCES

- Battelli, M.G., Polito, L., Bortolotti, M. and Bolognesi, A. 2016. Xanthine oxidoreductase-derived reactive species: Physiological and pathological effects. *Oxidative Medicine and Cellular Longevity*. 2016: 8.

- Berry, C.E. and Hare, J.M. 2004. Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. *The Journal of Physiology*. 555: 589-606.
- Borgers, M. and Thoné, F. 1992. Species differences in adenosine metabolic sites in the heart. *Histochemical Journal*. 24: 445-452.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 72: 248-254.
- Cherin, T., Catbagan, M., Treiman, S. and Mink, R. 2006. The effect of normothermic and hypothermic hypoxia-ischemia on brain hypoxanthine phosphoribosyl transferase activity. *Neurological Research*. 28: 831-836.
- Chu, S.Y., Cashion, P. and Jiang, M. 1989. A new colorimetric assay for purine nucleoside phosphorylase. *Clinical Biochemistry*. 22: 357-362.
- Citterio-Quentin, A., Mahmoudi, A.E., Perret, T., Conway, A., Ryan, A., Beringer, A., Lachaux, A. and Boulieu, R. 2019. Red blood cell IMPDH activity in adults and children with or without azathioprine: Relationship between thiopurine metabolites, ITPA and TPMT activities. *Basic Clinical and Pharmacological Toxicology*. 124: 600-606.
- Dorion, D., Zhong, A., Chiu, C., Forrest, C.R., Boyd, B. and Pang, C.Y. 1993. Role of xanthine in reperfusion injury of ischemic skeletal muscles in the pig and human. *Journal of Applied Physiology*. 75: 246-255.
- Dudzinska, W., Hlynczak, A.J., Skotnicka, E. and Suska, M. 2006. The purine metabolism of human erythrocytes. *Biochemistry*. 71: 467-475.
- Fukuda, T., Goebel, J., Thogersen, H., Maseck, D., Cox, S., Logan, B., Shebotie, J., Seikaly, M. and Vinks, A.A. 2011. Inosine monophosphate dehydrogenase (IMPDH) activity as a pharmacodynamics biomarker of mycophenolic acid effects in pediatric kidney transplant recipients. *Journal of Clinical Pharmacology*. 51: 309-320.
- Giannattasio, S., Gagliardi, S., Samaja, M. and Marra, E. 2003. Simultaneous determination of purine nucleotides, their metabolites and β -nicotinamide adenine dinucleotide in cerebellar granule cells by ion-pair high performance liquid chromatography. *Brain Research Protocol*. 10: 168-174.
- Giordano, F.J. 2005. Oxygen, oxidative stress, hypoxia and heart failure. *Journal of Clinical Investigation*. 115: 500-509.
- Giuliani, P., Zuccarini, M., Buccella, S., Rossini, M., D'Alimonte, I., Ciccarelli, R., Marzo, M., Marzo, A., Di Lorio, P. and Caciagli, F. 2016. Development of a new HPLC method using fluorescence detection without derivatization for determining purine nucleoside phosphorylase activity in human plasma. *Journal of Chromatography B*. 1009-1010: 114-121.
- Glander, P., Braun, K.P., Hambach, P., Bauer, S., Mai, I., Roots, I., Waiser, I., Fritsche, L., Neumayer, H.H. and Budde, K. 2001. Non-radioactive determination of inosine 5'-monophosphate dehydrogenase (IMPDH) in peripheral mononuclear cells. *Clinical Biochemistry*. 34: 543-549.
- Han, Y.H., Xia, L., Song, L.P., Zheng, Y., Chen, W.L., Zhang, L., Huang, Y., Chen, G.Q. and Wang, L.S. 2006. Comparative proteomic analysis of hypoxia-treated and untreated human leukemic U937 cells. *Proteomics*. 6: 3262-3274.
- Hedstrom, L. 2009. IMP dehydrogenase: Structure, mechanism and inhibition. *Chemical Review*. 109: 2903-2928.
- Hosek, B., Bohacek, J. and Kautská, J. 1986. The effect of hypoxia on the activity of purine nucleoside phosphorylase in rats. *Biomedical and Biochemical Acta*. 45: 281-284.
- Huang, Y., Hickey, R.P., Yeh, J.L., Dadak, A., Young, L.H., Johnson, R.S. and Giordano, F.J. 2004. Cardiac myocyte-specific HIF-1 α deletion alters vascularization, energy availability, calcium flux, and contractility in the normoxic heart. *Federation of American Societies for Experimental Biology Journal*. 18: 1138-1140.
- Jacobs, A.E., Oosterhof, A. and Veerkamp, J.H., 1988. Purine and pyrimidine metabolism in human muscle and cultured muscle cells. *Biochemical and Biophysical Acta*. 30: 130-136.
- Jain, J., Almquist, S.J., Ford, P.J., Shlyakhter, D., Wang, Y., Nimmesgern, E. and Germann, U.A., 2004. Regulation of inosine monophosphate dehydrogenase type I and type II isoforms in human lymphocytes. *Biochemical Pharmacology*. 67: 767-776.
- Jonsson, C.A. and Carlsten, H. 2001. Inosine monophosphate dehydrogenase (IMPDH) inhibition in vitro suppress lymphocyte proliferation and the production of immunoglobulins, autoantibodies and cytokines in splenocytes from MRL/pr/lpr mice. *Clinical and Experimental Immunology*. 124: 486-491.
- Kayyali, U.S., Donaldson, C., Huang, H., Abdelnour, R. and Hassoun, P.M. 2001. Phosphorylation of xanthine dehydrogenase/oxidase in hypoxia. *Journal of Biological Chemistry*. 276: 14359-14366.
- Kelley, E.E., Hock, T., Khoo, N.K.H., Richardson, G.R., Johnson, K.K., Powell, P.C., Giles, G.I., Agarwal, A., Lancaster, J.R. and Tarpey, M.M. 2006. Moderate hypoxia induces xanthine oxidoreductase activity in arterial endothelial cells. *Free Radical Biology and Medicine*. 15: 952-959.
- Lahat, N., Bitterman, H., Engelmayer-Goren, M., Rosenzweig, D., Weiss-Cerem, L. and Rahat, M.A. 2011. Reduced TIMP-2 in hypoxia enhances angiogenesis. *American Journal of Physiology- Cell Physiology*. 300: C557-C566.
- López-Cruz, R.I., Pérez-Milicua, M.B., Crocker, D.E., Gaxiola-Robles, R., Bernal-Vertiz, J.A., de la Rosa, A., Vázquez-Medina, J.P. and Zenteno-Savín, T. 2014. Purine nucleoside phosphorylase and xanthine oxidase activities in erythrocytes and plasma from marine, semiaquatic and terrestrial mammals. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*. 171: 31-35.
- Maiuolo, J., Oppedisano, F., Gratteri, S., Muscoli, C. and Mollace, V. 2016. Regulation of uric acid metabolism and excretion. *International Journal of Cardiology*. 213: 8-14.
- Moriwaki, Y., Yamamoto, T. and Higashino, K. 1999. Enzymes involved in purine metabolism: a review of histochemical localization and functional implications. *Histology and Histopathology*. 14: 1321-1340.
- Nagao, H., Nishizawa, H., Tanaka, Y., Fukata, T., Mizushima, T., Furuno, M., Bamba, T., Tsushima, Y., Fujishima, Y., Kita, S., Funahashi, T., Maeda, N., Mori, M., Fukusaki, E. and Shimomura, I. 2018. Hypoxanthine secretion from human adipose tissue and its increase in hypoxia. *Obesity*. 26: 1168-1178.
- Nelson D.L., Lehninger A.L., Cox M.M. *Lehninger Principles of Biochemistry*. 5th ed. Ch WH Freeman and Company; New York, NY, USA: 2008. pp. 882-900.
- Nyhan, W.L. 2005. Disorders of purine and pyrimidine metabolism. *Molecular Genetics and Metabolism*. 86: 25-33.

- Pang, B., McFaline, J.L., Burgis, N.E., Dong, M., Taghizadeh, K., Sullivan, M.R., Elmquist, C.E., Cunningham, R.P. and Dedon, P.C. 2012. Defects in purine nucleotide metabolism lead to substantial incorporation of xanthine and hypoxanthine into DNA and RNA. *Proceedings of the National Academy of Sciences USA*. 109: 2319-2324.
- Poss, W.B., Huecksteadt, T.P., Panus, P.C., Freeman, B.A. and Hoidal, J.R. 1996. Regulation of xanthine dehydrogenase and xanthine oxidase activity by hypoxia. *American Journal of Physiology*. 270: 941-946.
- Raivio, K. O., Saksela, M. and Lapatto, R. 2001. Xanthine oxidoreductase – Role in human pathophysiology and in hereditary xanthinuria. En: *The metabolic and molecular bases of inherited diseases*. Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds.), 8th edn. pp 2639-2652. New York: McGraw Hill.
- Ramírez, J.M., Folkow, L.P. and Blix, A.S., 2007. Hypoxia tolerance in mammals and birds: From the wilderness to the clinic. *Annual Review of Physiology*. 69: 113-143.
- Rao, P.N., Walsh, T.R., Makowka, L., Rubin, R.S., Weber, T., Snyder, J.T. and Starzl, T.E. 1989. Purine nucleoside phosphorylase: a new marker for free oxygen radical injury to the endothelial cell. *Hepatology*. 11: 193-198.
- Schomaker, S., Warner, R., Bock, J., Johnson, K., Potter, D., Winkle, J.V. and Aubrecht, J. 2013. Assessment of emerging biomarkers of liver injury in human subjects. *Toxicological Sciences*. 132: 276-283.
- Schumacker, P.T. 2011. Lung cell hypoxia: Role of mitochondrial reactive oxygen species signaling in triggering responses. *Proceedings of the American Thoracic Society*. 8: 477-484.
- Soñanez-Organis, J.G., Vázquez-Medina, J.P., Zenteno-Savín, T., Aguilar, A., Crocker, D.E. and Ortiz, R.M. 2012. Prolonged fasting increases purine recycling in post-weaned northern elephant seals. *Journal of Experimental Biology*. 215: 1448-1455.
- Pérez-Milicua, M., Zenteno-Savín, T., Crocker, D.E. and Gallo-Reynoso, J.P. 2015. Hypoxanthine-guanine phosphoribosyltransferase and inosine 5'-monophosphate dehydrogenase activities in three mammalian species: aquatic (*Miroiunga angustirostris*), semi-aquatic (*Lontra longicaudis*) and terrestrial (*Sus scrofa*). *Frontiers in Physiology*. 6: 1-7.
- Terada, L.S., Guidot, D.M., Leff, J.A., Willingham, I.R., Hanley, M.E., Piermattei, D. and Repine, J.E. 1992. Hypoxia injures endothelial cells by increasing endogenous xanthine oxidase activity. *Proceedings of the National Academy of Sciences U.S.A.* 89: 3362-3366.
- Traut, T.W. 1994. Physiological concentrations of purines and pyrimidines. *Molecular Cell and Biochemistry*. 140: 1-22.
- Rocha, S. 2007. Gene regulation under low oxygen: holding your breath for transcription. *Trends in Biochemical Sciences*. 32: 389-397.
- Vannoni, D., Bernini, A., Carlucci, F., Civitelli, S., Pietro, M.C.D., Leoncini, R., Tabucchi, A., Tanzini, G. and Marinello, E. 2004. Enzyme activities controlling adenosine levels in normal and neoplastic tissues. *Medical Oncology*. 21: 187-195.
- Velasco-Martínez, I.C., Hernández-Camacho, C.J., Méndez-Rodríguez, L.C. and Zenteno-Savín, T. 2016. Purine metabolism in response to hypoxic conditions associated with breath-hold diving and exercise in erythrocytes and plasma from bottlenose dolphins (*Tursiops truncatus*). *Comparative Biochemistry and Physiology Part: A Molecular and Integrative Physiology*. 191: 196-201.
- Vethe, N.T., Mohamad, A., Reine, P.A., Andersen, A.M., Bremer, S., Line, P.-D., Rootwelt, H. and Bergan. 2014. Simultaneous quantification of IMPDH activity and purine base in lymphocytes using LC-MS/MS: Assessment of biomarker responses to mycophenolic acid. *Therapeutic Drug Monitoring*. 36: 108-118.
- Vorbach, C., Harrison, R. and Capecchi, M.R. 2003. Xanthine oxidoreductase is central to the evolution and function of the innate immune system. *Trends in Immunology*. 24: 512-517.
- Wagner, P.D. 2008. The biology of oxygen. *European Respiratory Journal*. 31: 887-890.
- Wheaton, W.W. and Chandel, N.S. 2011. Hypoxia. 2. Hypoxia regulates cellular metabolism. *American Journal of Physiology and Cellular Physiology*. 300: C385-C393.
- Wu, J., Bond, C., Chen, P., Chen, M., Li, Y., Shohet, R.V. and Wright, G. 2015. HIF-1 α in the heart: remodeling nucleotide metabolism. *Journal of Molecular and Cellular Cardiology*. 82: 194-200.
- Yin, J., Ren, W., Huang, X., Deng, J., Li, T. and Yin, Y. 2018. Potential mechanism connecting purine metabolism and cancer therapy. *Frontiers in Immunology*. 9: 1697.
- Zar, J.H. 2009. *Biostatistical analysis* 5th ed. Prentice Hall, Upper Saddle River, NJ.
- Zhang, Y., Morar, M. and Ealick, S.E. 2008. Structural biology of the purine biosynthetic pathway. *Cellular and Molecular Life Sciences*. 65: 3699-3724.