Article

Effects of purine nucleotide administration on purine nucleotide metabolism in brains of heroin-dependent rats

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Heroin is known to enhance catabolism and inhibit anabolism of purine nucleotides, leading to purine nucleotide deficiencies in rat brains. Here, we determined the effect of exogenous purine nucleotide administration on purine nucleotide metabolism in the brains of heroin-dependent rats. Heroin was administered in increasing doses for 9 consecutive days to induce addiction, and the biochemical changes associated with heroin and purine nucleotide administration were compared among the treated groups. HPLC was performed to detect the absolute concentrations of purine nucleotides in the rat brain cortices. The enzymatic activities of adenosine deaminase (ADA) and xanthine oxidase (XO) in the treated rat cortices were analyzed, and qRT-PCR was performed to determine the relative expression of ADA, XO, adenine phosphoribosyl transferase (APRT), hypoxanthine-guaninephosphoribosyl transferase (HGPRT), and adenosine kinase (AK). Heroin increased the enzymatic activity of ADA and XO, and up-regulated the transcription of ADA and XO. Alternatively, heroin decreased the transcription of AK, APRT, and HGPRT in the rat cortices. Furthermore, purine nucleotide administration alleviated the effect of heroin on purine nucleotide content, activity of essential purine nucleotide metabolic enzymes, and transcript levels of these genes. Our findings therefore represent a novel, putative approach to the treatment of heroin addiction.

Uniterms: Purine nucleotide/quantitative analysis. Purine nucleotide/effects/brain rats. Heroin analysis. Adenosine deaminase analysis. Xanthine oxidase analysis

INTRODUCTION

Opiates, such as morphine and heroin, are illegally used as recreational drugs worldwide, and can significantly impair the user's physical and mental health (Chu *et al.*, 2009; Li *et al.*, 2014). Opioid dependence is a significant public health concern, underscoring the need for effective treatment options (Kresina, Bruce, Mulvey, 2013; Nutt, King, Phillips, 2010). Therefore, the exploration of the effects of repeated exposure to these compounds is important for understanding the mechanisms of drug dependency and developing new treatment options.

Properly regulated purine nucleotide (PN) metabolism is necessary for normal brain function (Franke, 2011). Imbalances in purine nucleotide levels lead to a variety of human diseases (Burhans, Weinberger,

2007; El-Hattab, Scaglia, 2013; Kimura *et al.*, 2003). Studies have shown that morphine and heroin can enhance the oxidation of xanthine and hypoxanthine in the lobus temporalis, lobus frontalis, and lobus parietalis of rats (Liu *et al.*, 2007; Yang *et al.*, 2006). Uric acid (UA) is the final oxidation product of purine metabolism in humans and higher primates, and changes in UA levels may reflect the catabolism of purine nucleotides (Liang, Clark, 2004). Furthermore, it has been reported that morphine may increase UA concentrations in the corpus striatum, as well as in serum and extracellularly, *in vitro* (Enrico *et al.*, 1997; Sumathi, Niranjali Devaraj, 2009). In patients where morphine was administered intracerebroventricularly for cancer-related pain, the UA levels in cerebrospinal fluid samples increased significantly (Goudas *et al.*, 1999). Morphine may also increase ATP catabolic products, including nucleotides and oxypurines, in BALB/c mouse strains (Di Francesco *et al.*, 1998).

Previously, we have shown that opioids may lead to nucleotide metabolism disorders. Rats were administered

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morphine (ip) over a period of 7 days in doses of 20, 30, 45, 55, 65, 85, and 95 mg/kg, respectively, to induce morphine addiction (Liu *et al*., 2007). Rats also received heroin (ip) over a period of 9 days in doses of 3, 3.45, 4.35, 5.7, 7.5, 9.75, 18.68, 17.16, and 21.12 mg/kg, respectively, to induce heroin addiction (Yang *et al*., 2006). Significant increases in UA concentration in plasma were observed in the rats following both morphine and heroin administration. The enzymatic activities of ADA and XO were found to be enhanced significantly in the brain and other organs of rats administered heroin, and morphine was shown to increase ADA and XO gene expression in the rat brain cortex (Liu *et al.*, 2007; Yang *et al.*, 2006).

Our experiments using rat C6 glioma cells also revealed that the gene expression of HGPRT and AK, two key enzymes in the purine nucleotide salvage pathway, are down-regulated by morphine, indicating that morphine inhibits purine nucleotide anabolism (Liu, Hong, Zhao, 2003). To further clarify the effects of heroin on purine nucleotide metabolism *in vivo*, we measured the absolute content of purine nucleotides in rat brain cortices by HPLC, and found that the levels of AMP and GTP are significantly reduced in rats administered heroin, resulting in a purine nucleotide deficiency (Li *et al.*, 2011). Therefore, we hypothesize that purine nucleotide deficiency in the brain may be one of the biochemical mechanisms of heroin dependence. Thus, the goals of this study were to confirm that purine nucleotide metabolism disorders are induced by heroin, and to clarify whether purine administration can counteract the biochemical changes caused by heroin.

MATERIAL AND METHODS

Animals and Drug Treatment

Adult, male Wistar rats (n = 40, 180 ± 20 g) were obtained from the Jilin University Animal Laboratory (Changchun, China), housed under controlled environmental conditions with free access to food and water, and randomly divided into four groups. Each group included 10 animals, and heroin was administered twice a day at 12-h intervals. Group I served as the control group and rats in this group received normal saline (ip) for 9 days. Rats in groups II, III, and IV were treated with heroin, heroin+PN, and PN alone, respectively. Heroin was administrated (ip) in increasing daily doses of 0.5, 0.75, 1.25, 2, 3, 4.25, 5.75, 5.75, and 5.75 mg/kg, for 9 days (Li *et al.*, 2009). PN was administrated by gavage at a constant dose of 30 mg/kg (AMP+GMP; in equimolar concentrations), 2 h before the administration of heroin for 9 days. On the morning of the 10th day, animals were killed via decapitation following ether-induced narcosis. Their brains were rapidly removed and thoroughly washed to remove the excess blood in ice-cold saline. The cortices were removed on a pre-cooled plate, snap frozen in liquid nitrogen, and stored at -80 °C. All experimental procedures were approved by the Experimental Animal Ethics Committee of Jilin University.

Reagents

Heroin (99% purity) was obtained from the Public Security Office of Jilin province, China. Adenine and guanine nucleotides (ATP, AMP, GTP, and GMP) and the ion-pair reagent tetra butyl ammonium hydrogen sulfate (TBAHS) were purchased from Sigma. HPLC-grade acetonitrile (ACN) and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals used in this study were of analytical grade. Distilled water, filtered with a Milli-Q Academic Ultrapure Water System (Millipore, Bedford, MA, USA), was used to prepare the standard solutions and HPLC mobile phases. ADA and XO detection kits, and Coomassie blue protein detection kits, were purchased from Jinsite Biotechnology Co. Ltd. (Nanjing, China). PrimeScript RT Reagents Kits and SYBR PrimeScript RT-PCR kits were purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China).

Content detection of purine nucleotides

Samples were analyzed with an Agilent 1100 HPLC system equipped with an autosampler, using Agilent Zorbsx SB-C18 (4.6×150 mm; 3.5 μm) columns. The mobile phase contained 10% methanol and 220 mmol/L KH_2PO_4, normalized to pH 6.5 with TBAHS. The flow rate of HPLC elution was 500 μL/min. The temperature of the column was 27±1 °C. The eluate was monitored at 254 nm, with a sample size of 20 μL (Chen *et al.*, 2007).

Cortex tissue (0.5 g) was removed from liquid nitrogen and pulverized in 4.5 mL cold, physiologic saline with a tissue pulping machine, at 12,000 rpm in an ice-water bath. The tissue samples were sonicated in 0.5 mL 6% HClO solution for 30 s in an ice-water bath, adjusted with TBAHS (4 mol/L) to pH 7.0, then centrifuged. The supernatant was filtered through 0.2 pm 8-mm cellulose filters. The samples were injected directly into the HPLC column for PN measurement. By comparing retention times with pure GMP, AMP, GTP, and ATP, all compounds were chromatographically identified. Linearity was tested using five known standard solutions of increasing concentrations. Calibration curves were calculated by linear regression analysis of the peak

area vs. concentration for GMP, AMP, GTP, and ATP. The concentrations of GMP, AMP, GTP, and ATP in the rat cortex samples were calculated by plotting the peak area against the known standard concentrations.

Enzyme assays

The tissue homogenate was centrifuged at 3000 rpm for 10 min at 4 °C. The sedimented fraction was discarded, leaving only the supernatant, which represented 10% of the total tissue homogenate. Protein content was estimated using Coomassie blue protein detection kits. The activity of ADA and XO in the cortex samples were estimated using Guisti colorimetric and colorimetric detection kits, respectively (Li *et al*., 2011; Yang *et al*., 2006).

Evaluation of ADA, XO, HGPRT, APRT, and AK gene expression by qRT-PCR

Gene-specific primers were designed and a BLAST (NCBI) search was performed to ensure their specificity to the target mRNA transcript. The primers were synthesized by the Jinsite Corporation (Nanjing, China). The primer sequences for ADA, XO, HGPRT, APRT, AK, and β-actin are summarized in Table I.

Total RNA was extracted with Trizol (Gibco BRL, Grand Island, NY, USA), normalized to a final concentration of 500 ng/µL, and reverse transcribed using the PrimeScript RT Reagents Kit according to the manufactures instructions. qRT-PCR analyses were performed on an ABI Prism 7000 Real Time PCR System (Applied Biosystems, Foster City，CA，USA), in a final volume of 20 µL, containing 10 µL SYBR Premix Ex Taq, 0.4 µL of each primer, 0.4 µL ROX Reference Dye, 2 µL RT product, and 6.8 µL dH$_2$O, using the SYBR PrimeScript RT-PCR kit. The following qRT-PCR cycling conditions, recommended by the manufacturer, were used: 95 °C for 30 sec, followed by 40 cycles at 95 °C for 5 sec and 60 °C for 31 sec. All samples were run in 96-well optical plates in duplicate. The cycle threshold values (Ct) were calculated and exported to Microsoft Excel for analysis. Quantification was performed by experimental determination of Ct, defined as the cycle at which the fluorescence exceeds 10 times the standard deviation of the mean baseline emission for the early cycles (Collantes-Fernandez *et al.*, 2002). The Pfaffl mathematical model was used for qRT-PCR data analyses (Pfaffl, 2001). β-actin was used as a reference gene.

Statistical analyses

The data were analyzed and expressed as mean ± SD. Statistical analyses were performed using SPSS 18.0. Analysis of Variance (ANOVA) and post hoc SNK-q tests were performed to examine differences between the groups. Significance was applied to values of 0.05 or 0.01 confidence.

RESULTS AND DISCUSSION

ADA and XO enzyme activity in rat cortices

The mean total protein content of the homogenates of the four groups (saline, heroin, heroin+PN, and PN)

TABLE I - Primer sequences for real-time PCR

mRNA		Sequence(5′→3′)	Amplified fragment base pairs (bp)	Genbank accession number
β-actin	Forward	AAGAGAGGCATCCTGACCCT	218	NM_031144
	Reverse	TACATGGCTGGGGTGTTGAA		
ADA	Forward	AAAGGAAGGCGTGGTGTA	165	NM_130399
	Reverse	ATGCCGAATGCTTGCTCT		
XO	Forward	AAGATCCGAAACGCCTGTGTG	132	NM_017154
	Reverse	GGAATCCTGGTGCGGTACAAAC		
HGPRT	Forward	TCCTCATGGACTGATTATGGACA	132	NM_012583
	Reverse	TAATCCAGCAGGTCAGCAAAGA		
APRT	Forward	TCCACGCACGGCGGCAAGAT	148	NM_001013061
	Reverse	AGGAGGCTGATACAGTGGG		
AK	Forward	GCCGAAGACAAGCACAAG	167	NM_012895
	Reverse	CCGAACTTATCTATCCCAAT		

were 6.2 ± 0.2, 6.3 ± 0.4, 6.4 ± 0.6 and 6.2 ± 0.3 g/L, respectively, which indicates that there was no significant difference in the total tissue protein concentrations (P >0.05). The mean ADA activity of the saline, heroin, heroin+PN, and PN groups was 6.5 ± 0.8, 8.8 ± 0.9, 6.6 ± 0.8 and 6.3 ± 0.7 g/L, respectively, indicating that the administration of heroin significantly increases cortex ADA activity as compared with that in the saline group (P<0.05; Figure 1), and that PN administration cancels out the increased ADA activity induced by heroin (P<0.05).

The mean XO enzyme activity of each of the four groups was 11.5 ± 1.2, 14.3 ± 1.2, 12.2 ± 1.0, and 11.7 ± 1.3 g/L, respectively. The XO activity of the heroin group was significantly higher than that of the saline group (P<0.05; Figure 1). The cortex XO activity of the heroin+PN group was significantly less than that of the heroin group (P<0.05). PN administration alone did not result in any significant difference in the examined enzyme activity as compared to the control (P>0.05).
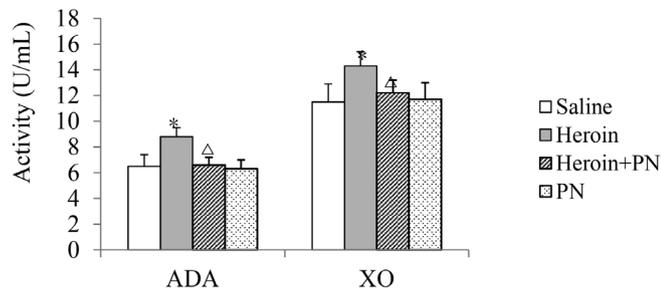

FIGURE 1 - ADA and XO activities in rat cortex. *P<0.05 vs. Saline. ^P<0.05 vs heroin.

ADA and XO are essential enzymes for purine catabolism. ADA irreversibly catalyzes the hydrolysis of adenosine to inosine. Inosine is then deribosylated by purine nucleoside phosphorylase, converting it to hypoxanthine (Cristalli *et al.*, 2001). XO catalyzes the oxidation of hypoxanthine and xanthine to UA. Previously, our research showed that the ADA and XO levels in many tissues of rats administered heroin *in vivo* are elevated, and the effects of heroin on the brain persist for a longer time than in other tissues (P<0.05; Yang *et al.*, 2006). These results indicate that heroin increases the ADA and XO levels in the cortex, and leads to increased purine nucleotide catabolism. This study shows that the administration of purine nucleotides, in combination with heroin, reduces ADA and XO levels dramatically (P<0.05) as compared with the group receiving only heroin, and indicates that increased purine nucleotide catabolism resulting from heroin administration, is negated by exogenous purine nucleotide administration.

mRNA transcript levels of key purine nucleotide metabolic enzymes

The ADA and XO mRNA levels in cortex tissue were significantly higher in the group administered with heroin alone, as compared with those in the saline-treated group (P<0.01). ADA and XO mRNA levels in the heroin+PN group, however, were significantly less than those in the heroin-treated group (P<0.05, P<0.01; Figure 2). As shown in Figure 3, AK mRNA levels were significantly lower in the group administered with heroin alone, as compared with that in the saline-treated control group (P<0.01). APRT and HGPRT mRNA levels were also significantly lower in the heroin-only group (P<0.01). The mRNA levels of HGPRT, APRT, and AK in the heroin+PN group were higher than those in the heroin-only group (P<0.05).
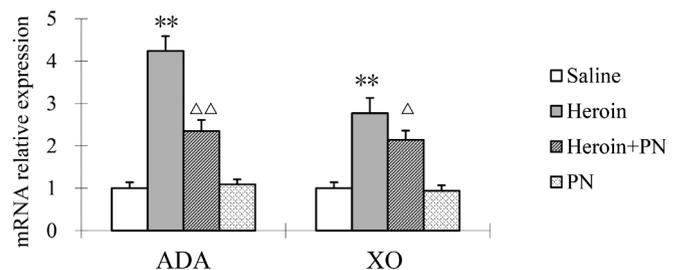

FIGURE 2 - Relative mRNA level of ADA and XO in rat cortex. Relative mRNA levels are normalized against β-actin. ** P<0.01 vs. Saline. ^P<0.05 vs Heroin. ^^ P<0.01 vs heroin.
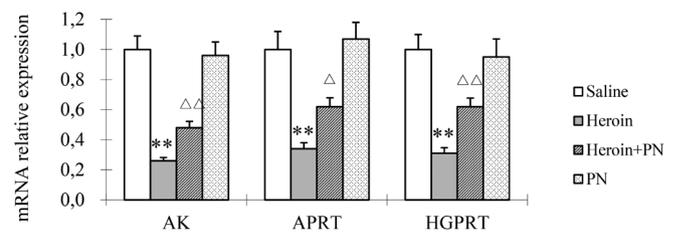

FIGURE 3 - Relative mRNA level of AK, APRT and HGPRT in rat cortex. Relative mRNA levels are normalized against β-actin. ** P<0.01 vs. Saline. ^P<0.05 vs heroin. ^^P<0.01 vs heroin.

Our data show significant increases in the ADA and XO mRNA levels in the cortices of rats administered with heroin alone, as compared with those in the control group that received only saline. Our results also show that these increases were counteracted by purine nucleotide administration. Furthermore, we found that the tendency of ADA and XO mRNA levels to change is correlated with the activity of the corresponding enzyme. Therefore, we conclude that the effect of heroin on purine nucleotide metabolism is dependent on the level of gene expression.

Purine nucleotides are synthesized either from simple precursors by *de novo* synthesis through energetic, multistep reactions, or assembled from free purine bases like guanine, hypoxanthine, and adenine, through nucleotide salvage pathways (Camici *et al.*, 2010). However, in some organs, including the brain, purine nucleotide synthesis relies exclusively on salvage pathways. Previously, our research showed that gene expression levels of HGPRT, AK, and APRT, essential enzymes in the purine nucleotide salvage pathway, are down-regulated by heroin both *in vivo* and *in vitro* (Li *et al.*, 2011; Liu *et al.*, 2007). HGPRT catalyzes the salvage of the purine bases hypoxanthine and guanine to produce nucleotides (Balasubramaniam, Duley, Christodoulou, 2014). APRT recycles adenine, produced by the polyamine pathway, to AMP. AK catalyzes the phosphorylation of adenosine to produce AMP (Camici *et al.*, 2010; Ipata *et al.*, 2011). As no HGPRT, APRT, or AK enzyme activity detection kits were available, we examined the expression levels of the HGPRT, APRT, and AK genes by qRT-PCR. Our results reveal that heroin inhibits the transcription of these three key enzymes involved in the purine nucleotide synthesis pathway in the rat cortex, however, the inhibitory effects of heroin on these enzymes could be negated with the administration of exogenous purine nucleotides.

Content detection of purine nucleotides

Liquid chromatographic analyses were performed as previously described. Figure 4 shows the retention time and area of the peak of the mixed reference solutions. Figure 5 shows the calibration curve obtained from cortex tissue for the purine nucleotides. Figure 6 shows the HPLC chromatogram of cortex tissue.

These results show that the proportion of AMP and GTP in the heroin-treated group decreased significantly as compared to those in the saline-treated group (Table II). However, purine nucleotide administration significantly reduced the magnitude of the AMP decrease induced by heroin ($P<0.05$), and alleviated the decrease in GTP, although not significantly.

To verify our hypothesis that heroin dependence results in purine nucleotide deficiency, we measured the ATP, GTP, AMP, and GMP contents in the treated rat cortices. The AMP and GTP content in the group administered heroin decreased significantly as compared with those in the saline-treated group, which indicates that heroin reduces the content of some purine nucleotides in brain. Purine nucleotide administration, however, alleviated the AMP deficit significantly ($P<0.05$), and also reduced the GTP deficiency, albeit to a lesser degree, in heroin-dependent rats.

Purine nucleotides play an important role in DNA synthesis, energy metabolism regulation, protein synthesis and function, and enzymatic activity. Purines are also essential components of many coenzymes, including NAD and Coenzyme A, and signaling molecules like cAMP (Duval *et al.*, 2013). However, the consequences of AMP and GTP deficiency in the brain are not yet clear. It has been reported that biochemical changes such as
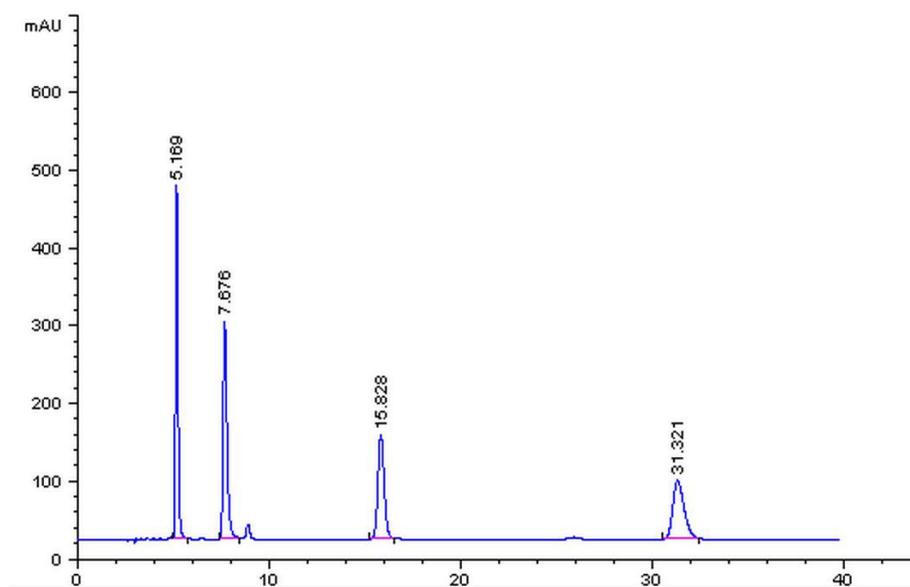

FIGURE 4 - HPLC chromatogram of mixed reference solution. From left to right the peaks are GMP, AMP, GTP, and ATP respectively.

GMP

AMP

$y = 53,145x$
$R^2 = 0,9943$

$y = 48,964x$
$R^2 = 0,9975$

GTP

ATP

$y = 41,923x$
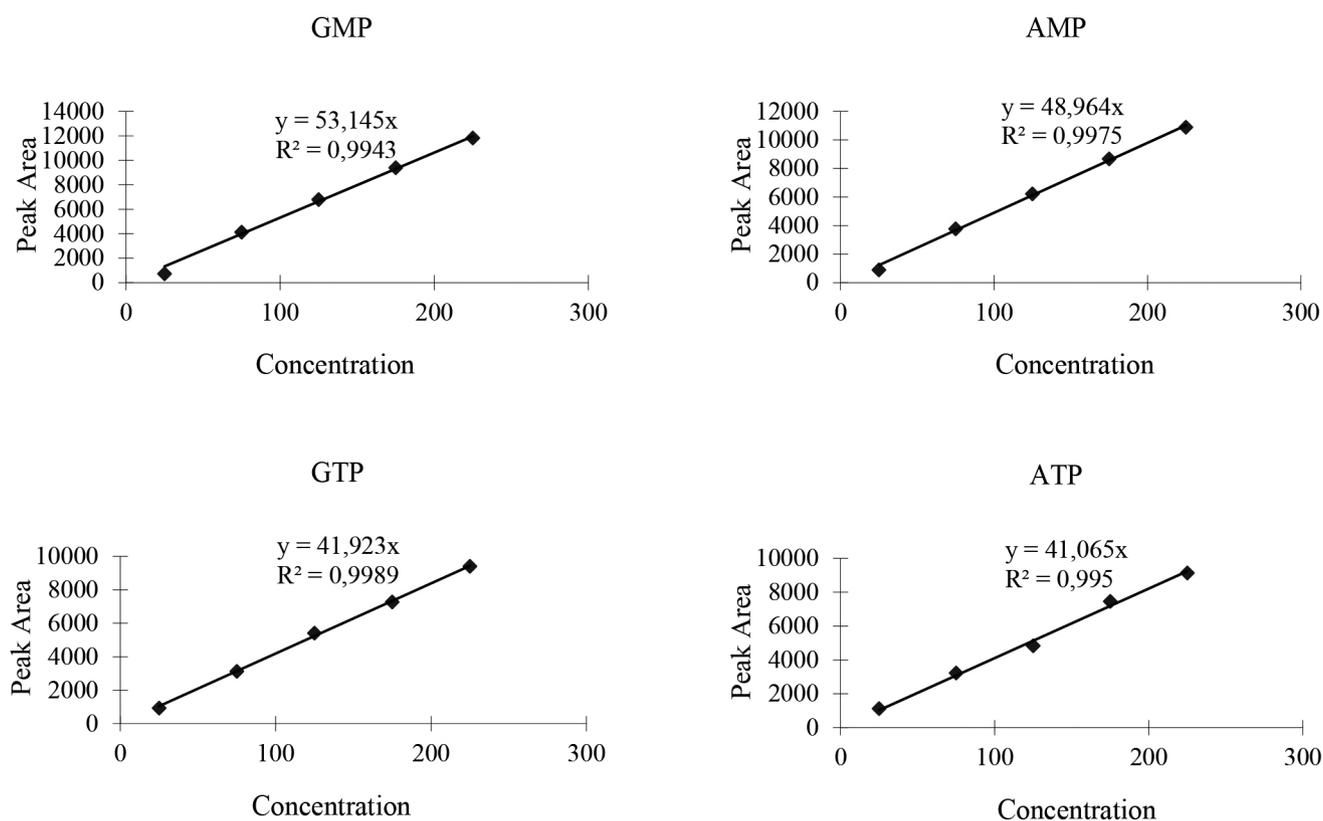$R^2 = 0,9989$

$y = 41,065x$
$R^2 = 0,995$

FIGURE 5 - Calibration curve obtained from cortex for purine nucleotides. Different concentrations of purine nucleotide (25 mg/mL, 75 mg/mL, 125 mg/mL, 175 mg/mL, 225 mg/mL) were used for the experiment.
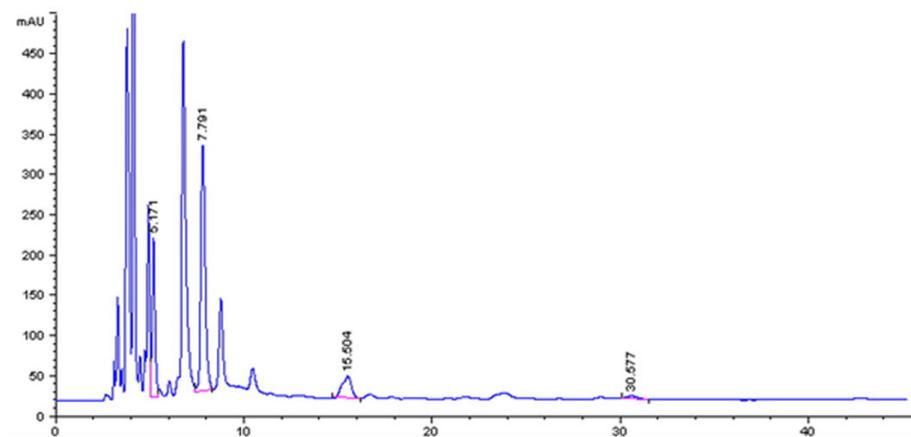
FIGURE 6 - HPLC chromatogram of cortex. From left to right the peaks are GMP, AMP, GTP, and ATP respectively.

TABLE II - The contents of GMP, AMP, GTP, ATP in rat cortex

	Saline	Heroin	Heroin+PN	PN
GMP (mg/mL)	37.35±2.59	36. 21±2.26	38.42±2.38	41.53±4.71
AMP (mg/mL)	87.78±3.92	49.04±3.11*	64.34±2.17△	92.69±5.12
GTP (mg/mL)	15.65±1.49	4.31±0.44*	5.26±0.83	17.83±2.45
ATP (mg/mL)	4.10±0.85	4.28±1.12	3.94±0.72	4.48±0.67

*$P<0.05$ vs Saline; △$P<0.05$ vs Heroin.

decreased GMP concentrations are associated with opioid dependence in humans (Mannelli *et al.*, 2009). It has also been shown that opioid use may have toxic effects on the central and peripheral nervous systems, leading to irreversible, pathological cellular changes. Furthermore, heroin and other opioids have been shown to inhibit the synthesis of DNA and RNA in brain tissues (Avella *et al.*, 2010; McLaughlin, Zagon, 2012). Our results indicate that the effect of heroin on the metabolism of purine nucleotides may result in purine nucleotide deficiency in the brain, representing a putative biochemical mechanism of heroin addiction. Our results also show that external compensation of purine nucleotides alleviates the deficiency in purine nucleotides in rat cortices caused by heroin, although purine nucleotide administration alone does not alter purine nucleotide concentrations, essential enzyme activity, or the mRNA levels of these key enzymes. Thus, purine nucleotide administration may represent a novel, effective approach to treating heroin addiction.

CONCLUSIONS

Our data show that heroin promotes catabolism and inhibits anabolism of purine nucleotides in the rat cortex, resulting in a purine nucleotide deficit. However, the administration of exogenous purine nucleotides can reverse these changes in nucleotide metabolism. Although our results are preliminary, they indicate that the relationship between purine nucleotide metabolism and opioid addiction deserves further study.

ACKNOWLEDGEMENTS

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