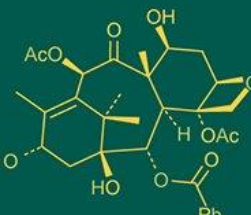
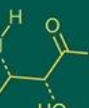
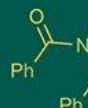
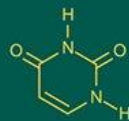


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## Bovine milk exosome encapsulated miR-148a, upregulates drug-metabolizing Cytochrome P450 genes epigenetically through DNA Methyltransferase 1 (DNMT1) in hepatocytes

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### Abstract

MicroRNAs are conserved non-coding small RNAs that can regulate post-transcriptional gene expression. Bovine milk contains bioactive, nanoscale extracellular vesicles known as exosomes. Among the microRNAs (miRNAs) encapsulated in these exosomes, miR-148a is the most abundant and has demonstrated potential to epigenetically modulate the human genome. The main findings of this study revealed that miR-148a is a potent inhibitor of DNA methylation and markedly upregulates CYP gene expression in HepG2 cells. It was also confirmed that miR-148a directly targets DNA methyltransferase (DNMT) 1, a key enzyme involved in epigenetic regulation, which influences the expression of cytochrome P450 (CYP) genes involved in hepatic drug metabolism. These findings suggest that miR-148a indirectly enhances the expression of specific CYP genes: CYP1A1, CYP1A2, CYP3A4, CYP2B6, CYP2C19, and CYP2D6 through negative regulation of DNMT1, thereby potentially enhancing hepatic metabolic function.

**Keywords:** miR-148a, CYP genes, DNMT1, HepG2

### Introduction

Milk contains a plethora of bioactive compounds that play pivotal roles in the signalling system. Recently, miRNAs have been identified as hidden bioactive components present in bovine milk (Rani *et al.*, 2017). Milk miRNAs can be present in free form in the skim milk fraction or packaged within carrier vesicles such as exosomes (Hata *et al.*, 2010). They are single-stranded small non-coding RNAs which are reported to be present in almost all body fluids, like saliva (Park *et al.*, 2009), blood plasma (Cortez & Calin, 2009), follicular fluid (Sang *et al.*, 2013), semen (Zubakov *et al.*, 2010), urine (Hanke *et al.*, 2010), etc. These are highly conserved across species, and it has recently been proposed that these miRNAs, encapsulated in exosomes, can be horizontally transferred between species. Exosomes mediate intercellular communication by interacting with target cell receptors via exosomal surface proteins or membrane protein fragments, or by fusing with the target cell membrane (Mathivanan & Simpson, 2010). So, it is important to know about bovine milk exosomes, their components, functions, absorption, and distribution, since bovine milk is a major source of human nutrition. It has been observed that biologically active cargo of exosomes carrying miRNAs can epigenetically regulate the genome. DNA methylation is the most extensively characterised and widely studied epigenetic modification. Epigenetic studies demonstrate that DNA methylation plays a critical role in gene regulation in HepG2 cells (Dannenberg & Edenberg, 2006). Also, NGS data showed the abundance of miR-148a among the 95 known miRNAs in cow milk exosomes (Rani *et al.*, 2017). Furthermore, the human liver is the primary organ responsible for metabolic detoxification, converting exogenous and endogenous substances into less toxic or metabolically inactive forms, primarily via Cytochrome P450 enzymes in the endoplasmic reticulum (Cribb *et al.*, 2005). Various studies have examined the role of exosomal miRNA cargo as a biomarker of liver injury (Yang *et al.*, 2011). Cytochrome genes belonging to the CYP1, CYP2, and CYP3 families are primarily responsible for drug metabolism in the phase I oxidation system (Zanger & Schwab, 2013).

Tsuchiya *et al.* (2006) were the first to show that drug-metabolizing CYP genes are regulated by miRNA in vitro. It has been reported that miR-148a act as a potential modifier of the human genome by targeting DNMT1 (Melnik & Schmitz, 2017). DNMT1 is the most prevalent DNA methyltransferase in mammalian cells and has a high affinity for hemi-methylated DNA, thereby contributing to the regulation of epigenetic states. The methylation status of CpG sites is maintained by DNA methyltransferase activity. Also, it has been reported that certain cytochrome genes, such as CYP1A2, CYP3A4, CYP2B6, CYP2C19, and CYP2D6, are regulated by DNMT1 (Nakamura *et al.*, 2017). Till now, there is no information on how bta-miR-148a is involved in regulating CYP genes in liver cells.

This study aims to investigate the impact of bovine milk exosome-derived miR-148a and a synthetic miR-148a mimic on DNMT1 expression and the transcriptional regulation of key Cytochrome P450 (CYP) genes in HepG2 cells, a widely used human hepatocellular carcinoma cell line.

## 2 Materials and methods

**2.1 Cell culture:** HepG2 cells were obtained from the National Centre for Cell Science (NCCS), Pune, India. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 2 mM glutamine, non-essential amino acids and antibiotics (100 U/ml of penicillin, 100 µg/ml of streptomycin and 25 µg/ml of amphotericin B) and were incubated at 37 °C with 5% CO<sub>2</sub>. Subculturing was performed in a T-25 cm<sup>2</sup> flask at a seeding density of  $1 \times 10^6$  cells/flask with medium refreshed every 2-3 days. Cells between passages 29-55 were used for the experiments. The Trypan blue method was used to assess cell viability.

**2.2 Exosome isolation, quantification and characterisation:** Milk samples for exosome isolation were collected from healthy, early lactating, non-pregnant Sahiwal cows maintained at the National Dairy Research Institute, Karnal. Approximately 50 mL of milk per animal was transferred to sterile tubes and transported on ice to the laboratory. Initial centrifugation of milk at 1200g for 10 minutes at 4°C was performed using a Hermle Z 446 K centrifuge (Germany) to remove fat and cellular debris. The resulting supernatant was subjected to two consecutive centrifugation steps at 21,500 g for 30 minutes and 1 hour, respectively, at 4°C to obtain clarified whey. Exosomes were isolated from whey fraction using the MiRCURY<sup>TM</sup> exosome Isolation Kit (Qiagen CAT # 76743). Exosomal protein content was quantified using Pierce<sup>TM</sup> BCA protein Assay kit (CAT#REF 23227). Exosome size distribution was measured by dynamic light scattering using a Zetasizer instrument (Malvern Instruments Limited, UK). The average of three readings was used to determine particle size.

**2.3 Bioinformatics:** Predicted target genes of miR-148a were identified using TargetScan version 7.1. Each gene was prioritised based on its cumulative weighted context score ++ (CWCS), which reflects the overall suppressive effect anticipated from multiple miRNA binding sites. Target genes with CWCS greater than 0.5 were selected for further investigation. These genes were subsequently used to construct a protein-protein interaction (PPI) network using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins).

**2.4 Transfection of HepG2 cells:** Quantity of  $1 \times 10^5$  HepG2 cells per well was seeded in a 24-well plate. Exosomes were added at a concentration of 1 µg exosomal protein per ml of DMEM and incubated for 24 hours. Synthetic bta-miR-148a mimic (Ambion Cat #4464066) at 1 nM concentration was evaluated in combination with Xtreme GENE HP (Roche, Cat #06 366 236 001) at a 1:1 ratio. After 48 hours of culture, a lipid-miRNA complex was prepared using miR-148a mimic in Opti-MEM (Gibco by Life Technologies, U.S.), incubated for 20 minutes at room temperature, and then added dropwise to the cells. Six hours later, the medium was replaced, and cells were maintained for an additional 18 hours. For miR-148a mimic transfections, spent medium was removed and cells were treated either with fresh DMEM (control) or with the 1:1 miR-148a mimic-reagent complex. After 6 hours, the medium was refreshed, and cultures continued for 18 more hours. Cell viability was assessed using the MTT-based method (Sigma, Cat # M5655).

**2.5 Quantitative real-time PCR analysis:** TRI reagent (Sigma-Aldrich, Cat. # T9424) was used for total RNA isolation according to the Chomczynski and Sacchi (1987) protocol. An Implen Nanophotometer (Germany) was used to determine RNA concentration and purity. cDNA for miRNA was synthesised using the Qiagen miScript RT II kit (Cat # 218161), and cDNA for mRNA was prepared using the RevertAid First Strand cDNA Synthesis Kit (Cat. # K1622). Primers for real-time PCR analysis were designed using NCBI resources. Primer specificity was confirmed by aligning all sequences with GenBank entries using the Basic Local Alignment Search Tool (BLAST). Sequence homology between different species was determined using the ClustalW Multiple Sequence Alignment Program (BCM Search Launcher, <http://searchlauncher.bcm.tmc.edu>). Primers designed to amplify the housekeeping gene GAPDH and genes involved in hepatic drug metabolism (DNMT1, CYP1A1, CYP1A2, CYP3A4, CYP2B6, CYP2C19, CYP2D6) in the cultured HepG2 cells are listed in Table 2. For quantitative amplification of miRNA mimic and U6-in control, cDNA was mixed with 2x Quantitect SYBR Green PCR Master Mix, 10x miScript Universal Primer (Cat # 218161, Germany), and target miRNA primer, and incubated in an Applied Biosystems thermocycler under the PCR conditions specified in Table 3. mRNA expression of the listed genes was quantified by the relative quantification method, using GAPDH to control for experimental variation. qPCR conditions for mRNA are provided in Table 4. Fold change, calculated as  $2^{-\Delta\Delta Ct}$ , was used to represent results.

**2.6 Statistics:** Data were analysed using t-tests and one-way ANOVA. Mean  $\pm$  SEM from three independent experiments was used to present all experimental results. GraphPad Prism software version 5.1 was used for statistical analysis.

## 3 Results:

**3.1 in silico study:** Preliminary in silico bioinformatic analysis using TargetScan identified a high-affinity 8-mer binding site for bovine milk-derived exosomal miR-148a within the 3' Untranslated region (UTR) of the DNMT1 gene. Supporting this, STRING database analysis revealed that DNMT1 had the highest predicted interaction score among the candidate targets of miR-148a (Fig 1). DNMT1 was selected as a target gene of miR-148a, and the CYP genes

CYP1A1, CYP1A2, CYP3A4, CYP2B6, CYP2C19, and CYP2D6 were selected to study epigenetic regulation in the liver detoxification process.

**3.2 Transfection with milk exosomes:** The size of cow milk exosomes was confirmed with Zetasizer as 62.52 nm with a polydispersity index < 0.5 (Fig 2). Exosomal protein concentration values were extrapolated from a standard curve generated with serially diluted BSA standards (Fig 3). Exosome-treated HepG2 cells showed a significant increase ( $p<0.05$ ) in miR-148a expression compared to the control (Fig 4). After 24 hours of treatment with exosomes in HepG2 cells, DNMT1 expression was significantly decreased ( $p<0.05$ ) compared with the control, as shown in Fig 5. Expression of drug metabolizing genes such as CYP1A1, CYP1A2, CYP3A4 and CYP2B6, CYP2C19 and CYP2D6 was found to be significantly increased ( $p<0.05$ ) as compared to control (Fig 6a-6f).

**3.3 Transfection with miR-148a mimic:** Standardisation was done using miR-148a mimic in two different ratios with transfection reagent, i.e., 1:1 and 2:1 (Fig 7). HepG2 cells

transfected with miR-148a mimic and transfection reagent in a 1:1 ratio were used for further experiments. After 24 hours of transfection with miR-148a mimic in HepG2 cells, gene expression of DNMT1 was analysed by real-time PCR. Results showed a significant ( $p<0.05$ ) decrease in DNMT1 gene expression in mimic-transfected cells compared with control, as shown in Fig 8. After 24 hours of transfection with miR-148a mimic in HepG2 cells, the gene expression of the drug-metabolizing genes CYP1A2, CYP2B6, CYP1A1, CYP3A4, CYP2D6, and CYP2C19 was analysed by qPCR. Results showed a significant ( $p<0.05$ ) increase in the gene expression of CYP1A2, CYP2B6, CYP1A1, CYP3A4, CYP2D6, and CYP2C19 in mimic-transfected cells compared with control, as shown in Fig (9a-9f).

**3.4 Effect on cell proliferation:** MTT analysis results showed a significant ( $p<0.05$ ) decrease in the viability of cells when treated with cow milk exosomes for 24 hours as compared to the control (Fig 10). miR-148a mimic-transfected cells showed a significant ( $p<0.05$ ) increase in cell viability as compared to the control, as shown in Fig 11.

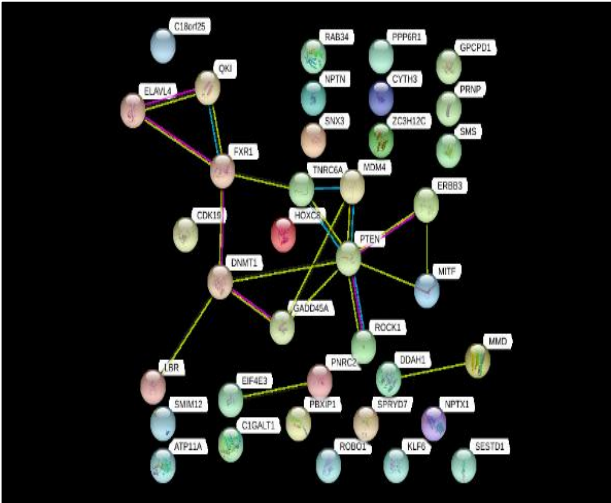


Fig 1: Network Analysis using STRING software

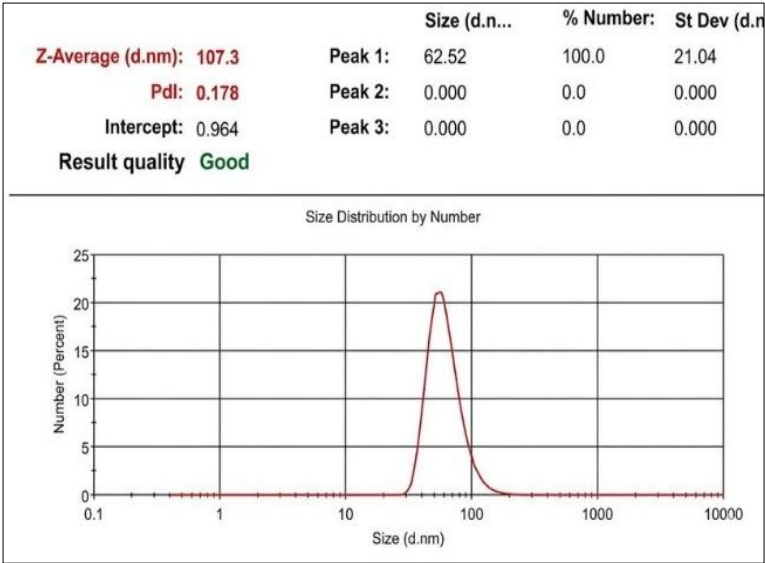


Fig 2: Characterisation of cow milk exosomes

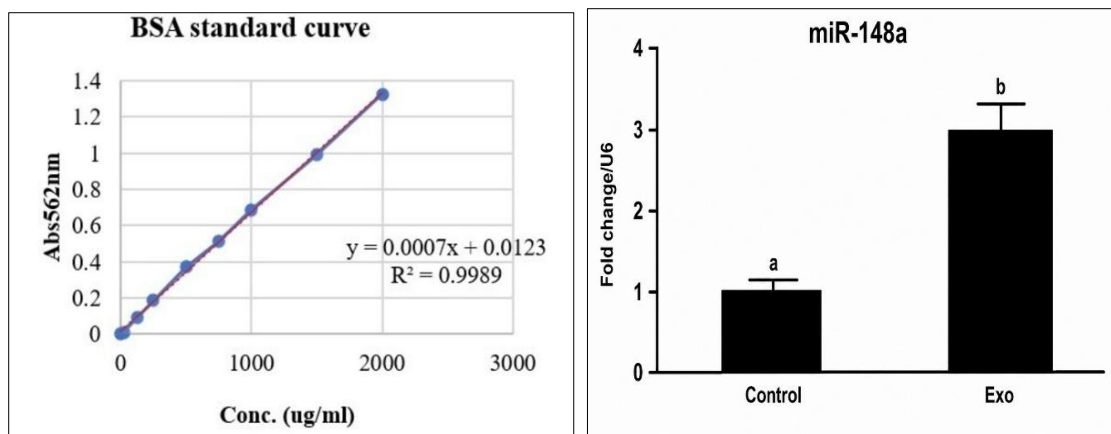


**Fig 3:** BSA standard curve for exosomal protein quantification, **Fig 4:** Effect of milk-derived exosomes on miR-148a expression in HepG2 cells

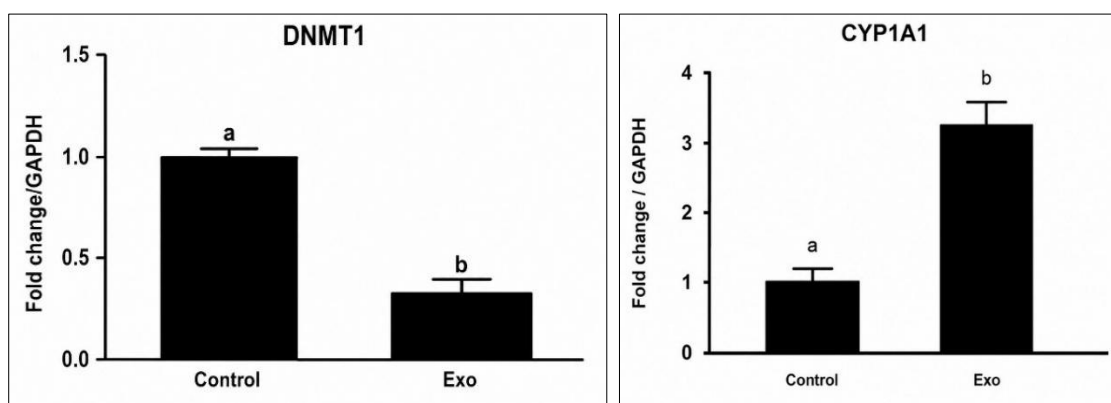


**Fig 5:** Effect of bovine milk exosomes on DNMT1 expression in HepG2 cells, **Fig 6:** (a)

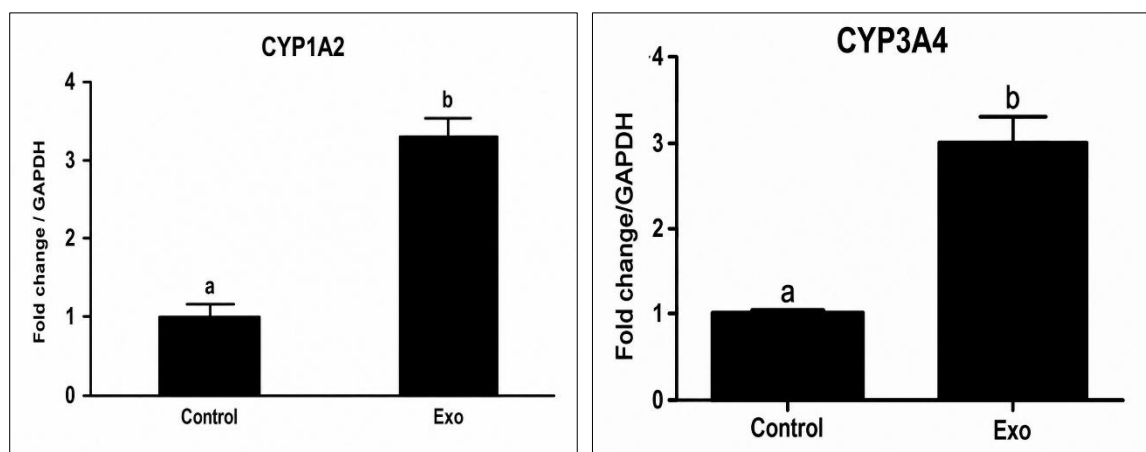


**Fig 6:** (b)

**Fig 6:** (c)

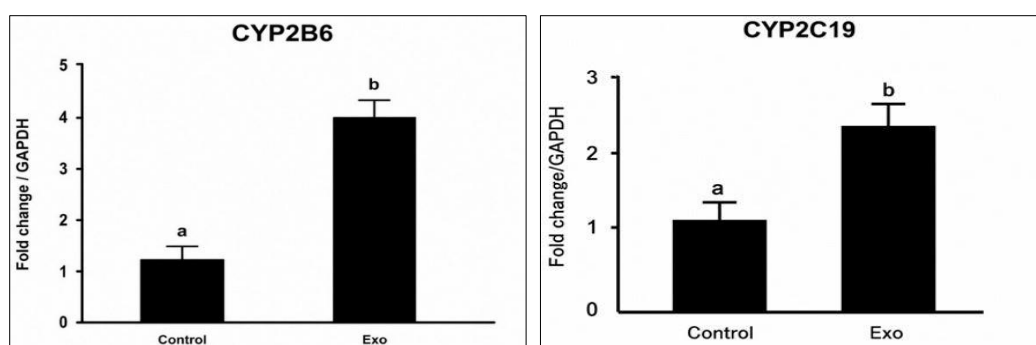


**Fig 6:** (d)

**Fig 6:** (e)

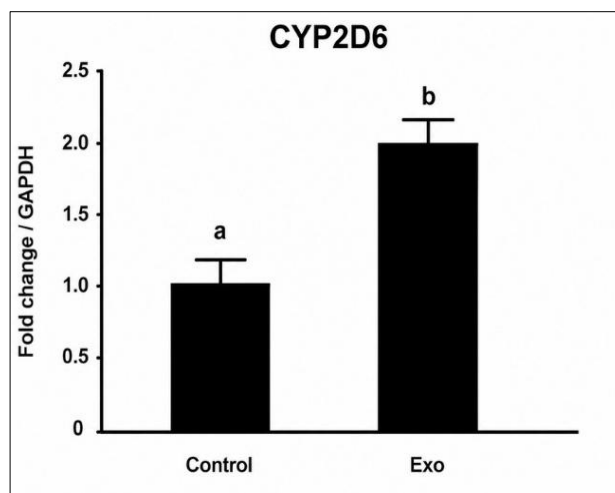


Fig 6 (f)

**Fig 6:** Effect of milk exosomes on Cytochrome P450 gene expression in HepG2 cells (Fig 6a-6f)

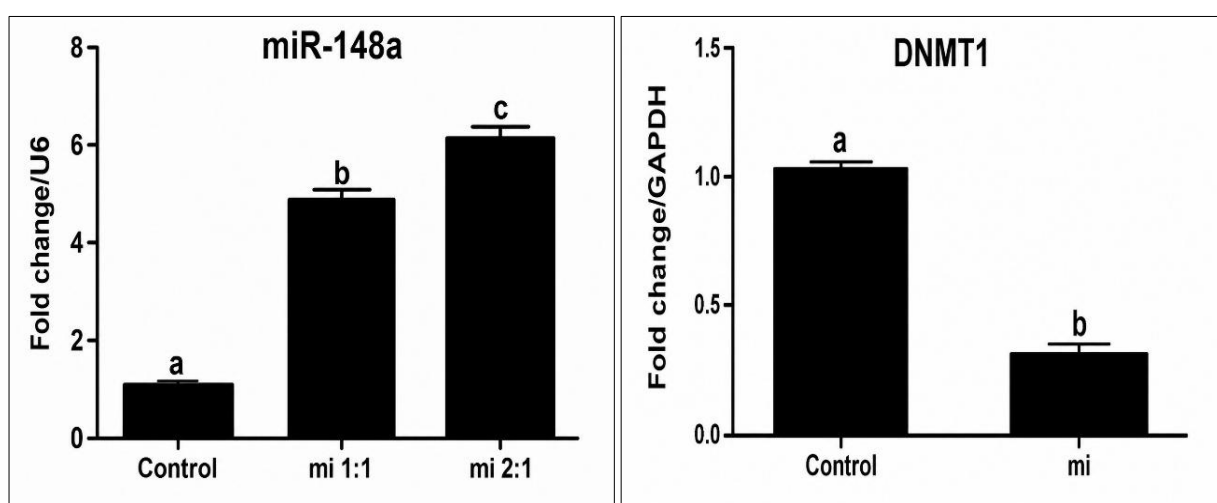


**Fig 7:** Optimisation of miRNA mimic transfection in HepG2 cells, **Fig 8:** Effect of miR-148a mimic on DNMT1 expression in HepG2 cells

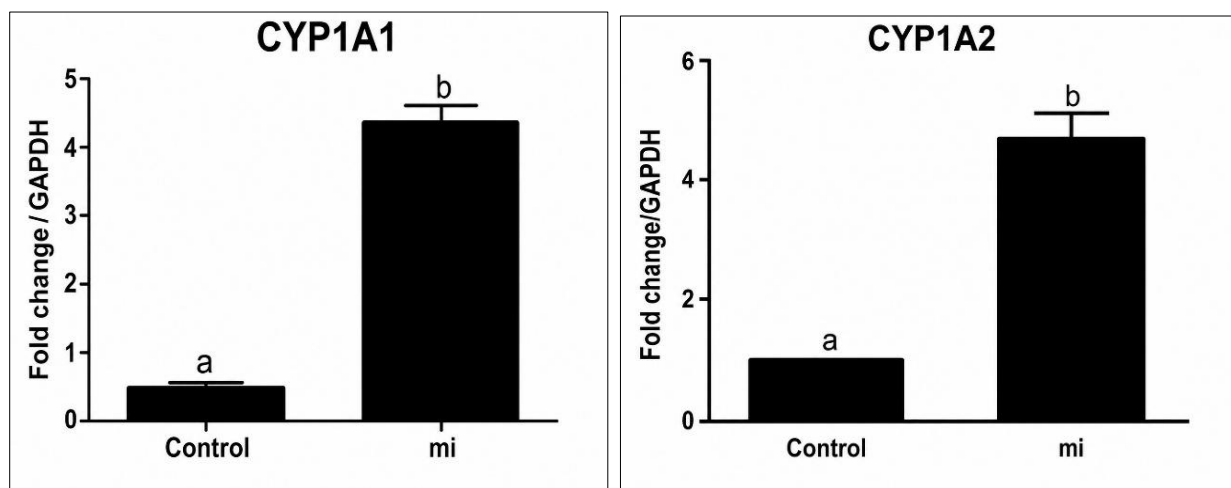


Fig 9 (a)

Fig 9 (b)

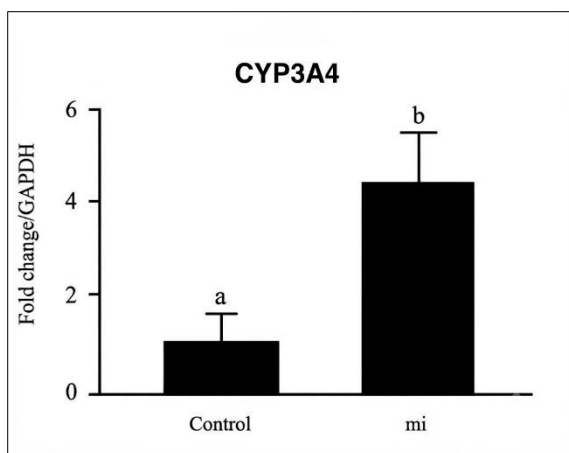


Fig 9 (c)

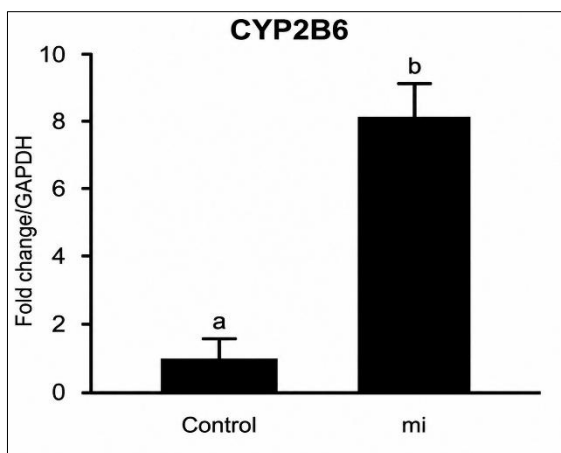


Fig 9 (d)

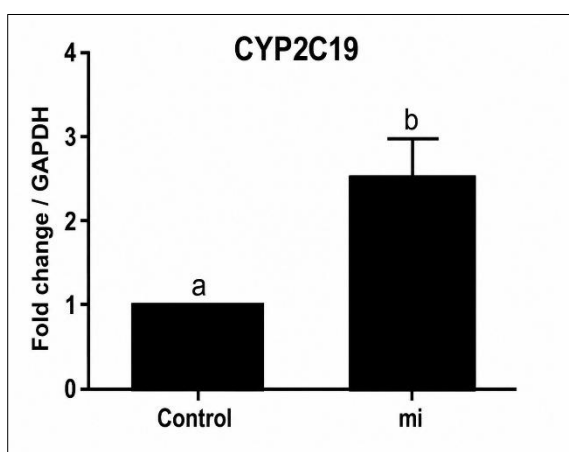


Fig 9 (e)

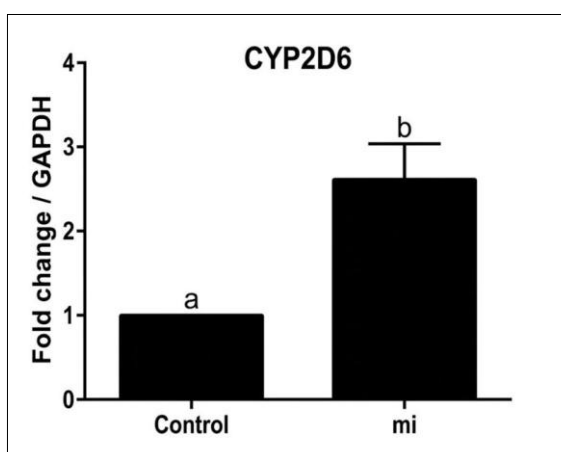


Fig 9 (f)

Fig 9: Effect of miR-148a mimic on CYP genes in HepG2 cells (Fig 9a-9f)

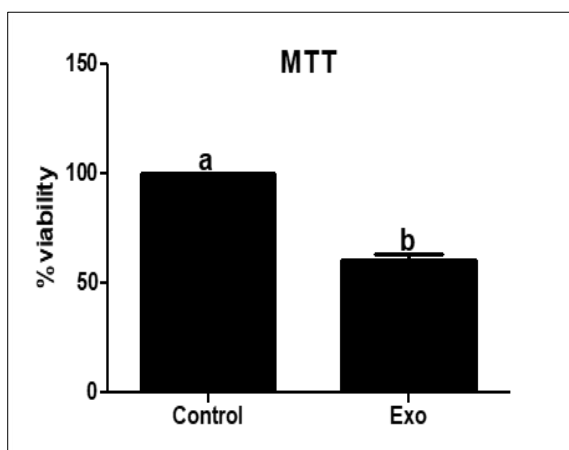
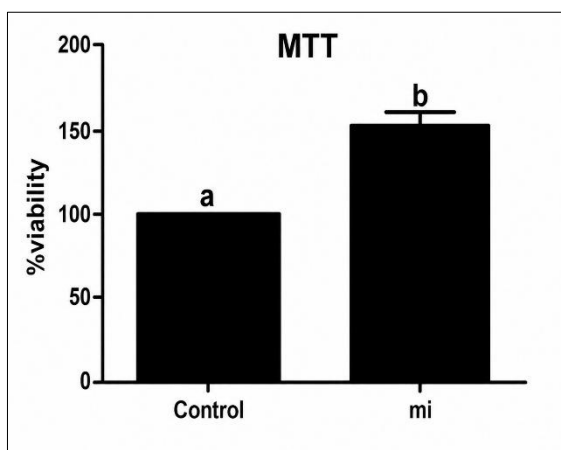


Fig 10: Effect of bovine milk exosomes on HepG2 cell viability, Fig 11: Effect of miR-148a mimic on HepG2 cell viability



#### 4. Discussion

**4.1 In silico analysis:** Preliminary in silico bioinformatic analysis using TargetScan identified a high-affinity 8-mer binding site for bovine milk-derived exosomal miR-148a within the 3' Untranslated region (UTR) of the DNMT1 gene, suggesting a potential mechanism of post-transcriptional regulation. Supporting this, STRING database analysis revealed that DNMT1 had the highest predicted interaction score among the candidate targets of miR-148a. Based on these predictions and previous literature, DNMT1 was prioritised for experimental validation. Indeed, previous

studies have consistently reported DNMT1 as a validated target of miR-148a, supported by tools including TargetScan, Miranda, and PicTar, indicating miR-148a's capacity to modulate DNMT1 expression (Zang *et al.*, 2016).

Given the liver's central role in drug metabolism and detoxification, the current study aimed to investigate the regulatory effects of bovine milk exosome-derived miR-148a and a synthetic miR-148a mimic on DNMT1 expression and the transcriptional regulation of key Cytochrome P450 (CYP) genes in HepG2 cells, a widely used human hepatocellular carcinoma cell line.

**4.2 Effect of bovine milk exosomes on DNMT1 and CYP gene expression:** Treatment of HepG2 cells with bovine milk exosomes resulted in significant downregulation of DNMT1 and concurrent upregulation of multiple CYP genes. These observations align with findings suggesting that milk exosomes modulate gene expression via the miR-148a-DNMT1 axis, thereby altering DNA methylation (Reif *et al.*, 2019). Since DNMT1 is known to silence CYP genes through promoter methylation (Nakamura *et al.*, 2017), its suppression may lead to hypomethylation and reactivation of these genes. This provides a potential mechanism by which milk-derived exosomal miR-148a influences hepatic gene expression.

**4.3 miR-148a mimic recapitulates DNMT1 suppression and CYP gene activation:** Transfection of HepG2 cells with a synthetic miR-148a mimic resulted in a significant decrease in DNMT1 expression, accompanied by a notable increase in the expression of several Cytochrome P450 (CYP) genes, indicating that miR-148a modulates hepatic drug metabolism indirectly through suppression of DNMT1. These results align with the previous studies demonstrating that miR-148a isolated from both skim and fat fractions of milk downregulates DNMT1 expression (Melnik and Schmitz, 2017), and with similar observations in human colon epithelial cells (Golan-Gerstl *et al.*, 2019). DNMT1 is a key methyltransferase responsible for maintaining CpG methylation, thereby repressing gene expression (Robert *et al.*, 2003). Its downregulation may result in hypomethylation and reactivation of silenced genes, including those involved in xenobiotic metabolism. The present findings are also consistent with a study that demonstrated that DNMT1 epigenetically regulates multiple CYP genes, including CYP1A2, CYP2B6, CYP2C19, CYP2D6, and CYP3A4 (Nakamura *et al.*, 2017).

Among the upregulated genes, CYP2B6 exhibited the highest fold change, consistent with previous data showing that transfection with a miR-148a mimic directly enhances CYP2B6 expression by targeting the 3' UTR (Luo *et al.*, 2021). Collectively, these results suggest that miR-148a, whether delivered via exosomes or as a synthetic mimic, plays a regulatory role in hepatic drug-metabolizing pathways by targeting DNMT1, thereby modulating CYP gene expression.

**4.4 Differential effects of milk exosomes and miR-148a mimic on HepG2 cell proliferation:** Transfection of HepG2 cells with a synthetic miR-148a mimic significantly increased cell proliferation, suggesting a potential pro-proliferative role of miR-148a in hepatic cells. This observation is supported by previous findings showing that silencing miR-148a-3p in HEK-293 cells increased proliferation, whereas adding milk-derived exosomes to these cells suppressed this effect (Reif *et al.*, 2019). Furthermore, bta-miR-148a has been shown to promote hepatocyte proliferation, corroborating the current results (Jung *et al.*, 2016). These contrasting effects suggest that the overall impact of milk exosomes on hepatocytes is complex and likely involves multi-miRNA interactions beyond miR-148a alone.

## 5 Summary

We found that both bovine milk exosomes and synthetic miR-148a mimic similarly regulate DNMT1 and Cytochrome P450 genes, although their effect on cell proliferation differed, likely due to additional factors in exosomes. Our findings demonstrate an unprecedented regulatory mechanism of CYP gene expression in HepG2 cells and underscore the potential of milk exosomal miR-148a as a bioactive dietary component that influences epigenetic regulation and liver function. Further investigation is required to clarify the mechanism by which miR-148a regulates CYP genes in HepG2 cells.

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## Author's contribution

All authors contributed to designing the study. Anika managed the data collection, material preparation, and analysis. Anika wrote the first draft of the manuscript, and all authors commented on it. All authors approve the final manuscript.

## Compliance and ethical standards

Conflict of interest: None. No ethical approval was required for this study.

**Table 1:** Target genes of miR-148a identified by Target Scan version 7.1

| Gene symbol | Ensembl Transcript ID | Description                                       |
|-------------|-----------------------|---------------------------------------------------|
| RASAL 1     | ENST00000546530.1     | RAS protein activator like 1 (GAP1 like)          |
| PTEN        | ENST00000371953.3     | Phosphatase and tensin homolog                    |
| DSTYK       | ENST00000367160.4     | Dual serine/threonine and tyrosine protein kinase |
| ACVR 1      | ENST00000263640.3     | Activin A receptor, type I                        |
| DNMT 1      | ENST00000340748.4     | DNA (cytosine-5)-methyltransferase 1              |
| TMSB 10     | ENST00000233143.4     | Thymosin beta 10                                  |

**Table 2:** Primer sequences used for real-time PCR

| S. No. | Gene name | Primer name: sequence (5'-3')                            | Product length | Accession no. |
|--------|-----------|----------------------------------------------------------|----------------|---------------|
| 1      | CYP1A1    | F.P. TGGAGACCTTCCGACACTCT<br>R.P. ACAAGACACAACGCCCTT     | 109            | NM_000.499.5  |
| 2      | CYP1A2    | F.P. TCCTTCGCTACCTGCCTAAC<br>R.P. CCCTTCTTGCTGTGCTGAAC   | 160            | NM_000761.5   |
| 3      | CYP3A4    | F.P. ACAGTCATCCACAATTCCAACA<br>R.P. GGTCCACTTCCAAAGGGTGT | 132            | NM_008421.1   |
| 4      | CYP2B6    | F.P. CCACCCTAACACCCATGACC<br>R.P. GTCCCAGGTGTACCGTGAAG   | 149            | NM_000767.5   |

|   |         |                                                         |     |                |
|---|---------|---------------------------------------------------------|-----|----------------|
| 5 | CYP2C19 | F.P. CTGCGGAATTTTGGGATGGG<br>R.P. ATGTTACCCACCCCTTGGTTT | 103 | NM_008384.3    |
| 6 | CYP2D6  | F.P. ACCTGTCATCGGTGCTGAAG<br>R.P. CACCGAGAAGCTGAAGTGCT  | 212 | NM_000106.6    |
| 7 | DNMT1   | F.P. TATCCGAGGAGGGCTACCTG<br>R.P. CTGCCATTCCCACTCTACGG  | 153 | NM_001130823.3 |

**Table 3:** Real-time PCR (miRNA) conditions for gene expression analysis

| Steps          | Temperature          | Time                                 |
|----------------|----------------------|--------------------------------------|
| Pre-incubation | 95°C                 | 15 min                               |
| Amplification  | 95°C<br>55°C<br>70°C | 15 sec<br>30 sec 40 cycles<br>40 sec |
| Melt-curve     | 95°C<br>70°C<br>97°C | 5 sec<br>1 min<br>Continuous         |
| Cooling        | 40°C                 | 30 sec                               |

**Table 4:** Real-time PCR (mRNA) conditions for gene expression analysis

| Steps          | Temperature          | Time                                 |
|----------------|----------------------|--------------------------------------|
| Pre-incubation | 95°C                 | 5 min                                |
| Amplification  | 95°C<br>60°C<br>72°C | 20 sec<br>15 sec 40 cycles<br>15 sec |
| Melt-curve     | 95°C<br>70°C<br>97°C | 5 sec<br>1 min<br>Continuous         |
| Cooling        | 40°C                 | 30 sec                               |

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