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厚生労働科学研究費補助金
食品の安全確保推進研究事業

食品中の化学物質 および 食品中の化学物質と医薬品との相互作用による肝毒性 ならびに発生毒性の新規評価系の構築

平成25年度　総括研究報告書

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研究代表者　中村　和昭　国立成育医療センター研究所薬剤治療研究部実験薬理研究長

研究要旨

食品中の化学物質と医薬品との相互作用は、医薬品との飲み合わせの観点から医薬品相互作用と同様の基準で評価される必要がある。しかし、食品中の化学物質について、医薬品との相互作用の観点に基づいたヒトを試験対象とした実験的な検討は十分に行われていない。また、化学物質暴露に対する脆弱性の高い胎児に対する食品中の化学物質曝露による影響は不明な点が多く、食品の安全の観点から、食品中の化学物質による発生毒性を明らかにする必要がある。本研究では、肝移植手術の際に生じる摘出肝から肝細胞の単離・培養を行い、ヒト肝細胞を用いた食品中の化学物質による肝機能への影響の評価系および食品中の化学物質単独および医薬品との併用による肝細胞毒性試験系を確立し、食品と医薬品の飲み合わせにおける食品の安全性評価系を構築することを目的とする。さらに、食品中の化学物質による発生毒性の評価系と食品中の化学物質と医薬品との相互作用による発生毒性が検討可能な評価系の構築を目指す。昨年度に引き続き、本年度においても健康食品として摂取される食品中の化学物質（ヒペルフォリン、ビロバリド、ギンコリドA、ギンコリドB、ギンコリドC）による発生毒性を検討し、さらに食品中の化学物質と医薬品の相互作用を考慮し得る発生毒性評価系の構築を試み、本研究成果を他の食品中の化学物質及び医薬品に応用するための知見を得た。

A. 研究目的

食品中の化学物質と医薬品との相互作用は、医薬品との飲み合わせの観点から医薬品相互作用と同様の基準で評価される必要がある。しかし、食品中の化学物質の発がん性等に関する動物や微生物等を用いた評価は行われているものの、ヒトを試験対象とした医薬品との相互作用の観点に基づいた実験的な検討は十分に行われていない。医薬品間の相互作用は代謝過程における相互作用が重要であり、そのほとんどが薬物代謝酵素チトクロムP450（CYP）の阻害または誘導に起因する。食品中の化学物質と医薬品との相互作用も同様の機構と考えられ、食品と医薬品の相互作用により有害事象を引き起こす代表的な例として、グレープフルーツジュースや西洋弟切草による CYP の阻害・誘導作用が挙げられる。医薬品間の相互作用は処方時に留意されるのに対し、食品と医薬品との相互作用は消費者の食生活に依るところが大きく、日常生活において予期せずに生じる可能性が高い。服用された薬物は体内へ吸収された後、その 90%以上が肝臓の CYP により代謝されることから、食品中の化学物質による CYP 発現変動は、服用する医薬品の薬物動態を変化させ、服用した医薬品の薬効の増減、あるいは副作用の増悪を引き起こす恐れがある。従って、食品中の化学物質による健康障害の予防の観点から、食品中の化学物質による CYP 発現への影響をはじめとする肝機能の評価系が必要である。また、肝臓は薬物代謝の主要な臓

器であるため、化学物質を含む薬物毒性を最も受けやすい臓器の一つである。したがって、肝臓に対する食品中の化学物質の毒性および食品と医薬品の複合摂取による肝毒性を評価することは、食品の安全を考える上でも、重要な課題である。

一方、化学物質の曝露に対する税癪性の高い胎児においては、化学物質による催奇形性等を検討することが、健全な胎児発育を考える上で重要である。妊婦における医薬品の摂取は、胎児への催奇形性の観点から処方時に留意されているが、食品中の化学物質による妊婦・胎児への影響については実験的な検証はほとんど行われておらず、また胎児への催奇形性が認められず安全だと考えられている医薬品においても、服用の際の食品の摂取状況によっては予期せぬ有害事象が生じる可能性を否定できない。

上述の観点から、本研究では、肝移植手術の際に生じる摘出肝から肝細胞の単離・培養を行い、ヒト新鮮肝細胞を用いた食品中の化学物質による肝機能への影響の評価系および食品中の化学物質単独あるいは医薬品との併用による肝細胞毒性試験系を検討し、食品と医薬品の飲み合わせにおける食品の安全性評価系の構築を試みる。さらに、食品中の化学物質による胎児への影響を検討するため、ES 細胞を用いた発生毒性試験法であるEmbryonic Stem Cell Test（EST 法）および肝細胞毒性試験系と EST 法との組み合わせによる、食品中の化学物質単独および医薬品との相互作用による胎児発育への影響について評価可能な新規試験系の構築を試みる。これまでにヒト肝細胞を用いた食品中の化学物質の安全性を評価する系は確立されておらず、本研究は新規の食品安全評価系の提案を念頭に、肝移植時に得られる摘出肝からの細胞単離に始まり胎児の発育における食品安全性の評価に至る一連の研究を遂行するものである。

B. 研究方法

1）ヒト新鮮肝細胞の継続的な分離・培養・保存

国立成育医療研究センター・病院臓器移植センター及び研究所先端医療開発室と連携し、肝移植時の摘出肝からの肝細胞の分離・保存を行った。

書面による同意あるいは保護者による代諾を頂いた提供者より、国立成育医療研究センターにて行われる小児生体肝移植手術の際生じる摘出肝組織（ドナー余剰肝組織及びレシピエント肝組織）のうち、移植術に用いずまた病理検査においても不要な廃棄予定の余剰肝組織を提供いただき、肝細胞の採取を行った。生体肝移植手術で摘出されたドナー肝組織は、手術室で執刀医が移植に必要な部位を確保した後の余剰廃棄部分を研究用として用いた。一方、摘出されたレシピエント肝組織においては、病理検査に必要な処理をした後の廃棄予定の組織を研究用として用いた。提供された肝組織は、一部を凍結保存あるいは組織観察用に固定し、残りは肝細胞分離に用いた。

肝細胞単離に当たっては、肝組織切断面より門脈もしくは中心静脈を確保し、血管腔へカニューレを挿入後血管周囲とともにカニューレを結索するこ

とにより固定し、灌流路を確保した後、37℃保温条件下でコラゲナーゼ液にて20 分間灌流し、組織構造を消化した。コラゲナーゼ液処理後、組織を分散し、ガーゼとナイロンメッシュにて濾過することにより大型の細胞塊を除去し、肝細胞懸濁液を得た。

２）食品中の化学物質による発生毒性試験

イチョウ葉エキスの成分であるビロバリド、ギンコリドAおよびギンコリドCの発生毒性をマウスES細胞を用いた発生毒性試験法である EST 法により検討した。ES 細胞および NIH/3T3 細胞を 3.2 × 10^4個/ウェルにて 96 ウェルプレートへ播種した。ビロバリド、ギンコリド A あるいはギンコリド C を添加し、10 日間培養した後、WST-8（DOJINDO）にて ES 細胞の細胞数を定量し、薬剤非添加条件の値を 100%としたときの細胞生存率を求めた。また、ビロバリド、ギンコリド A あるいはギンコリド C 暴露条件下で hanging drop 法により ES 細胞の胚様体を作成し、10 日間培養した後、ES 細胞を回収した。回収した ES 細胞から total RNA を抽出し、逆転写反応により cDNA を合成した。得られた cDNA を用いて、内胚葉、中胚葉および外胚葉分化マーカー遺伝子発現量を定量 PCR 法にて検討した。

３）食品中の化学物質と医薬品との相互作用による発生毒性試験

食品中の化学物質と医薬品との相互作用による発生毒性を検討するため、EST 法と肝細胞培養系を組み合わせた Hep-EST 法を用いて、催奇形性が知られているバルプロ酸（VPA）並びに西洋弟切草の活性成分であるヒペルフォリンあるいはギンコリド B との複合曝露による ES 細胞に対する細胞毒性を検討した。薬物代謝を担うヒト肝細胞として肝細胞株 HepG2 細胞、対照としてヒト繊維芽細胞 WI-38 細胞をミリセル 96 セルカルチャーインサートプレート（Millipore）のフィルタートレイへ 9 × 10^3個/ウェル播種した。翌日、マウス ES 細胞をミリセル 96 セルカルチャーインサートプレートのレシーバートレイへ 1.25 × 10^3 個/ウェル播種し、HepG2 細胞あるいは WI-38 細胞とともに培養した。培地には VPA およびヒペルフォリンあるいはギンコリド B を添加し、3 日目、5 日目にそれぞれ同濃度の薬物添加培地で培地交換を行い、7 日目に WST-8（DOJINDO）にて ES 細胞の細胞数を定量し、薬剤非添加条件の値を100%としたときの細胞生存率を求めた。同様に成熟細胞のモデルとして NIH/3T3 細胞と HepG2 細胞あるいは WI-38 細胞との共培養を試験化合物を添加した培地にて行い、7 日目の NIH/3T3 細胞の細胞生存率を求めた。

（倫理面への配慮）

本研究実施においては、対象患者個人のプライバシーをはじめとした人権擁護を最優先とし、危険性の排除や説明と理解（インフォームドコンセントおよびアセント）の徹底を図っている。

採取された肝組織を本研究に用いることは、国立成育医療研究センターの倫理審査委員会にて承認が得られている（「生体肝移植時に生じる余剰肝等からのヒト肝細胞の分離・培養・保存」（受付番号 385 平成 21 年 12 月 8 日承認）、「ヒト肝細胞・組織を用いた創薬研究および肝疾患・病態に関する基礎研究」（受付番号 396））。本研究に用いている肝組織は国立成育医療研究センター病院臓器移植センターにおいて主治医から説明を受け同意

を得た後に提供されたドナー余剰肝およびレシピエント摘出肝であり、通常は医療廃棄物として廃棄される組織である。肝組織は国立成育医療研究センター病院にて匿名化された後に国立成育医療研究センター研究所に搬送され、肝組織の一部の保存と肝細胞分離の処理を行っている。国立成育医療研究センター研究所においては、連結可能匿名化され管理番号のみ附された検体を受け入れている。本研究は、平成10年厚生科学審議会答申が定める「手術等で摘出されたヒト組織を用いた研究開発の在り方について」にも従い遂行した。

C. 研究結果

1）ヒト新鮮肝細胞の継続的な分離・培養・保存

本年度、成育医療研究センターにて実施した生体肝移植のうち、ドナー余剰肝およびレシピエント摘出肝より32例の肝検体より、肝組織の保存及び肝細胞の分離・培養・保存を行った。

2）食品中の化学物質による発生毒性試験系の確立

ビロバリド、ギンコリドAおよびギンコリドC曝露によるES細胞に対する毒性を評価した結果、ギンコリドC曝露においては、細胞毒性は認められなかったが、ビロバリドおよびギンコリドA曝露において細胞毒性が認められ、ビロバリドにおいてはギンコリドAよりも強い細胞毒性が認められた。しかし、いずれもヒペルフォリンによる細胞毒性と比べ、その毒性は100～1000倍程度低かった。また、ビロバリドおよびギンコリドA曝露によりES細胞分化誘導過程において、内胚葉マーカー遺伝子であるTTR (transthyretin) の発現低下が認められた。これらの結果は、ビロバリドおよびギンコリドはその毒性は低いものの、細胞毒性を有しており、また発生過程において内胚葉分化を抑制する可能性を示唆している。

3）食品中の化学物質と医薬品との相互作用による発生毒性試験

VPA 並びにヒペルフォリンあるいはギンコリドBとの複合曝露によるES細胞に対する細胞毒性を検討した結果、いずれの複合曝露においても、HepG2との共培養およびWI-38との共培養において、ES細胞に対する細胞毒性が観察された。しかし、ES細胞とHepG2細胞との共培養において、いずれの複合曝露においても、WI-38細胞との共培養と比べ細胞毒性の増悪は認められなかった。これまでの研究から、VPAは主にCYP2A6, 2B6および2C9により代謝され、活性中間体である4-en-VPAを生じることにより毒性を示すと考えられている。本研究により、ヒペルフォリンおよびビロバリドによるヒト肝細胞におけるCYP発現誘導作用を検討した結果、いずれの化学物質もCYP3A4の発現を惹起した。したがって、本研究による複合曝露の組み合わせにおいては、食品中の化学物質と医薬品との相互作用による細胞毒性の増悪は生じないと考えられたが、他の食品中の化学物質と医薬品との複合曝露に本検討を用いる有用性が示唆された。

D. 考察

「1）ヒト新鮮肝細胞の継続的な分離・培養・保存」において、手術摘出

検体より単離したヒト新鮮肝細胞を研究へ利用する体制の確立への知見が得られた。肝移植実施病院と研究機関が連携して、摘出後すぐに検体を研究に利用可能な体制を整えている国内研究機関は限られており、本研究は、今後の肝細胞を用いた研究における手術摘出検体の利用に関しての知見の蓄積に寄与するものである。ヒト肝細胞は創薬研究や毒性研究においてニーズが高く、本研究の知見を活用することにより、正常肝および疾患肝由来日本人肝細胞の研究利用に向けた体制構築が可能であると考えられる。

本年度研究成果「2）食品中の化学物質による発生毒性試験系の確立」においては、昨年度と合わせ、ヒペルフォリン、ビロバリド、ギンコリドAおよびCについて、EST法による食品中の化学物質による発生毒性の評価を行った。EST法は動物を用いないin vitro発生毒性試験系として注目されており、今後発生毒性の評価が必要とされる分野での活用が期待される。本研究における知見を、今後より精度の高い発生毒性評価系の構築へ応用することにより、食品中の化学物質による発生毒性試験系の構築が可能であると考えられた。

また、昨年度に引き続き、Hep-EST法を用いて、食品中の化学物質と医薬品との相互作用による発生毒性評価系の検討を行った。発生毒性を考える上では、母体による化学物質の吸収・代謝・排出等の薬物動態および胎盤移行性など様々な要因を考慮する必要がある。本研究では、薬物の薬効・副作用発現において最も重要な過程の一つである肝代謝をin vitro発生毒性試験系へ導入するため、Hep-EST法を考案し、その有用性を検証した。これにより、本研究「3）食品中の化学物質との医薬品の相互作用による発生毒性試験」の成果で示したような、食品中の化学物質と医薬品との相互作用による発生毒性評価系の確立が見込まれる。本評価系を他の化学物質へ応用することで、食品中の化学物質と医薬品の相互作用による発生毒性試験系の構築が可能であると考えられる。

E. 結論

本研究の成果により食品中の化学物質による肝機能への影響の評価系および食品中の化学物質単独および医薬品との併用による肝細胞毒性試験系ならびに発生毒性試験系の確立が可能であると考えられ、今後これら試験系をさらに発展させ、より精度の高い評価系の構築を目指すとともに、構築した評価系の活用・提供を通じ、本研究が食品の安全性確保の一助となる事を期待する。

F. 研究発表

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G. 知的財産権の出願・登録状況

該当なし

研究成果の刊行に関する一覧表

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Nakamura K, Aizawa K, Yamauchi J, Tanoue A.	Hyperforin inhibits cell growth and differentiation in mouse embryonic stem cells.	Cell Proliferation.	46	529-537	2013
Nakamura K*, Aizawa K, Nakabayashi K, Kato N, Yamauchi J, Hata K, Tanoue A.	DNA methyltransferase inhibitor zebularine inhibits human hepatic carcinoma cells proliferation and induces apoptosis.	PLOS ONE.	8	e54036	2013

LETTER FROM THE FRONTLINE

Hepatocyte Transplantation Using a Living Donor Reduced Graft in a Baby With Ornithine Transcarbamylase Deficiency: A Novel Source of Hepatocytes

Received October 29, 2013; accepted November 17, 2013.

TO THE EDITORS:

We performed hepatocyte transplantation (HT) in an 11-day-old infant with ornithine transcarbamylase deficiency (OTCD). We used cryopreserved hepatocytes prepared from remnant liver tissue, a byproduct of a hyper-reduced left lateral segment from living donor liver transplantation (LDLT). The patient exhibited hypothermia, drowsiness, and apnea at 3 days of age; these symptoms were accompanied by hyperammonemia (1940 μg/dL at maximum), although there were no abnormalities at birth or an obvious family history (Fig. 1). Further examinations confirmed that the hyperammonemia was the result of OTCD. Multimodal treatments, including alimentotherapy, medications, and continuous hemodiafiltration (CHDF), did not improve the patient's clinical state, and severe hyperammonemia attacks recurred. Because of the patient's small body size (2550 g) and the lack of an available liver donor, HT was indicated. Hepatocytes of the same blood type were chosen from an institutional repository of cryopreserved hepatocytes prepared from the remnant tissue of segment III from unrelated living donors. Thawed hepatocytes were transplanted twice at 11 and 14 days of age with a double-lumen catheter inserted into the left portal vein via the umbilical vein (Fig. 2). The amounts of transplanted hepatocytes were 7.4×10^7 and 6.6×10^7 cells/body, and the viability rates were 89.1% and 82.6%, respectively. The portal flow was kept stable at greater than 10 mL/kg/minute, and the pressure was maintained at less than 20 mm Hg during and after HT. The immunosuppressive treatment followed the same protocol used for LDLT with tacrolimus and low-dose steroids.[1] The patient was weaned from CHDF and the ventilator at 26 and 30 days of age, respectively, with a stable serum ammonia level of 40 μg/dL. The patient was ultimately discharged 56 days after HT. During the 3 months of follow-up, the baby did well with protein restriction (2 g/kg/day), medication for OTCD, and immunosuppression. No neurological sequelae related to hyperammonemia have been observed so far (Fig. 1).

DISCUSSION

For children with metabolic liver disease, HT is indicated as an alternative or bridge to liver transplantation.[2] HT is less invasive than liver transplantation and can be performed repeatedly. Limitations to the widespread application of HT include the poor availability of hepatocytes. Therefore, it is important to find new sources of high-quality hepatocytes. We previously prepared a repository of hepatocytes obtained from remnant liver tissue, a byproduct of hyper-reduced left lateral segmentectomy in LDLT.[1]

The cell donor was an unrelated volunteer with the same blood type who had previously undergone hyper-reduced left lateral segmentectomy. The main unit of segment II was used as a monosegmental liver graft for the primary recipient with end-stage liver disease, and the remnant was used to isolate hepatocytes with fully informed consent. The hepatocytes were isolated according to the collagenase perfusion method, as described elsewhere,[3] with Liberase MTF C/T GMP grade (Roche). All procedures were performed at our cell processing center according to a strictly controlled protocol based on good manufacturing practices. The total number of transplanted live hepatocytes was 1.4×10^8 cells/body; the ammonia removal rate was more than 200 fmol/cell/hour (203.4 and 265.4 fmol/cell/hour with the first and second injections, respectively). The dose was judged to be sufficiently high to obtain therapeutic effectiveness according to our theoretical background.[4]

This work was supported by a grant-in-aid from the National Center for Child Health and Development and the Highway Program for the Realization of Regenerative Medicine (Japanese Science and Technology Agency). This study protocol was approved by institutional review board in National Center for Child Health and Development (reference number 433).

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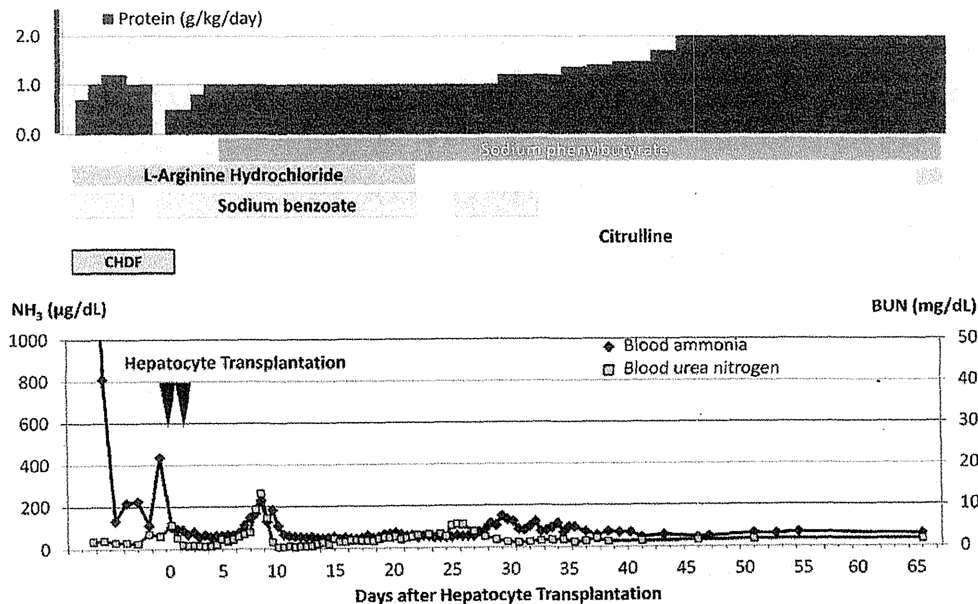

Figure. 1. Treatment schedule (top) and patient condition (bottom). The changes with time for blood ammonia and blood urea nitrogen are shown. The baby was delivered vaginally as a first child. At 3 days of age, hypothermia, low oxygen saturation, and, finally, respiratory arrest occurred. The patient was incubated and given artificial respiration. Concurrently, hyperammonemia (1940 μg/dL) was found, and continuous hemodiafiltration (CHDF) was started in addition to alimentotherapy (protein withdrawal) and medications. Whenever the administration of essential amino acids was restarted, the blood ammonia level became elevated, and at 9 days of age, despite the suspension of essential amino acid administration, the level increased up to 434 μg/dL. At 11 days of age, HT was performed for the first time, and it was performed for the second time at 14 days of age. After HT, amino acid intake was restarted along with the continuation of multimodal treatments, and blood ammonia was controlled well except for episodic increases. The patient was weaned from CHDF and the ventilator at 26 and 30 days of age, respectively, and the patient was ultimately discharged 56 days after HT.

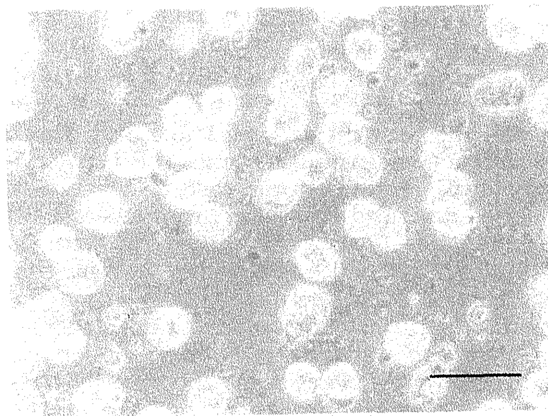

Figure. 2. Hepatocytes transplanted during the first injection. The cells showed a glazed and firm surface. The bar indicates 50 μm.

Because liver transplantation is approved as a treatment for end-stage hepatic failure, donor livers are preferentially allocated for organ transplantation and not for hepatocyte isolation. On rare occasions, the lack of appropriate donor-recipient matching (eg, infant donor livers) provides good-quality hepatocytes.[2] Fetal livers are also considered to be an alternative cell source, although ethical issues remain to be resolved. At present, we have little choice but to use marginal donor tissues, such as livers obtained from donors after cardiac death and organs with steatosis, fibrosis, or a long ischemia time. However, there are unfavorable issues related to the use of marginal donors, including low viability and vulnerability to cryopreservation. In this respect, the remnant liver tissue of hyper-reduction procedures used in LDLT has the same quality as that of left lateral segment grafts. As for availability, there are 5 cases of hyper-reduction per year at our institution on average.[5] The use of remnant liver tissues obtained from hyper-reduced LDLT procedures will, therefore, help to address the shortage of hepatocyte donors.

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Hyperforin inhibits cell proliferation and differentiation in mouse embryonic stem cells

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Abstract

Objectives: Hyperforin, a phloroglucinol derivative of St. John's Wort, has been identified as the major molecule responsible for this plant's products anti-depressant effects. It can be expected that exposure to St. John's Wort during pregnancy occurs with some frequency although embryotoxic or teratogenic effects of St. John's Wort and hyperforin have not yet been experimentally examined in detail. In this study, to determine any embryotoxic effects of hyperforin, we have attempted to determine whether hyperforin affects growth and survival processes of employing mouse embryonic stem (mES) cells (representing embryonic tissue) and fibroblasts (representing adult tissues).

Materials and methods: We used a modified embryonic stem cell test, which has been validated as an *in vitro* developmental toxicity protocol, mES cells, to assess embryotoxic potential of chemicals under investigation.

Results: We have identified that high concentrations of hyperforin inhibited mouse ES cell population growth and induced apoptosis in fibroblasts. Under our cell culture conditions, ES cells mainly differentiated into cardiomyocytes, although various other cell types were also produced. In this condition, hyperforin affected ES cell differentiation into cardiomyocytes in a dose-dependent manner. Analysis of tissue-specific marker expression also revealed that hyperforin at high concentrations partially inhibited ES cell differentiation into mesodermal and endodermal lineages.

Conclusions: Hyperforin is currently used in the clinic as a safe and effective antidepressant. Our data indicate that at typical dosages it has only a low risk of embryotoxicity; ingestion of large amounts of hyperforin by pregnant women, however, may pose embryotoxic and teratogenic risks.

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Introduction

Previous studies have demonstrated that many natural products with anti-bacterial, anti-inflammatory, and anti-tumour qualities, especially those related to chemoprevention and chemotherapy of various diseases, represent potential sources for new drug development (1–3). Extracts of St. John's Wort, *Hypericum perforatum*, have been used for centuries in traditional medicine, most notably for treatment of depression (4–6) and a number of its biologically active compounds have been isolated and characterized. These include naphthodianthrones, flavonoids and phloroglucinols such as hyperforin (6). Hyperforin has been identified as the major molecule responsible for the plant's anti-depressant effects. Its neurobiological consequences include neurotransmitter re-uptake inhibition, ability to increase intracellular sodium and calcium levels, recognized transient receptor potential activation and *N*-methyl-D-aspartic acid receptor antagonism (4–6). Hyperforin also displays several other biological properties of potential pharmacological interest, including anti-bacterial and anti-oxidant properties and an inhibitory effect on inflammatory mediators (5,6). In addition, hyperforin effectively inhibits proliferation of a number of mammalian cancer cell lines *in vitro* (4,5); it also induces apoptosis in K562 (chronic myeloid leukaemia) and U937 (acute myeloid leukaemia) cell lines through a caspase-dependent pathway (7,8).

St. John's Wort is an herbal medicine that has been shown to be effective in treating mild-to-moderate depression, which is common in women of childbearing

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years. Given significant numbers of unplanned pregnancies, it is expected that exposure to St. John's Wort during pregnancy occurs with some frequency. Despite fears related to maternal and foetal safety, embryotoxic or teratogenic effects of St. John's Wort and hyperforin have not, up to now, been experimentally examined in detail.

One powerful tool for studying these risks is in embryonic stem (ES) cell research. As ES cells have the ability to develop into differentiated cell types of endodermal, ectodermal and mesodermal lineages, ES cell lines are highly valuable for analyses of mutagenic, cytotoxic and embryotoxic effects of chemical compounds, *in vitro*. The ES cell test (EST), which employs mouse ES (mES) cells to assess embryotoxic potential of the tested chemicals, has been validated as an *in vitro* developmental toxicity test (9–11). In our laboratory, we developed an assay system based on the EST in our previous studies, and tested this *in vitro* system by evaluating embryotoxicity of known *in vivo* teratogens valproic acid, carbamazepine and fluoxetine (12–14). In the study described here, to experimentally estimate embryotoxic effect of hyperforin, we have attempted to determine whether hyperforin would affect prolferation and survival processes of mES cells (representing embryonic tissue) and fibroblasts (representing adult tissues) using this system. In addition, we sought to characterize embryotoxicity of hyperforin.

Materials and methods

Cell culture and differentiation

Mouse ES cells (R1; SCRC-1011) and NIH/3T3 cells (CRL-1658) were purchased from American Type Culture Collection (Manassas, VA, USA). NIH/3T3 cells were maintained at 37 °C in an atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. mES cells were maintained as previously described (12–14). ES cells were grown on gelatin-coated tissue culture dishes in standard ES cell culture medium [DMEM supplemented with 10% FCS, 2 mM glutamine, 0.1 mM non-essential amino acids, 0.1 mM βmercaptoethanol, 1000 U/ml LIF (Chemicon, Temecula, CA, USA), 50 U/ml penicillin G and 50 μg/ml streptomycin].

mES cell differentiation

R1 ES cell differentiation was carried out as described previously (12–14). In brief, ES cells were suspended in ES cell differentiation medium (DMEM supplemented with 20% FCS, 2 mM glutamine, 0.1 mM non-essential amino acids, 0.1 mM βmercaptoethanol, 50 U/ml penicillin G, and 50 μg/ml streptomycin) containing the appropriate dilution of hyperforin (Sigma-Aldrich, St. Louis, MO, USA), and cultured in hanging drops (500 cells/drop) as aggregates embryoid bodies, for 3 days. Embryoid bodies were then transferred to suspension culture dishes (Sumitomo Bakelite, Tokyo, Japan) and cultured for 2 days. These (n = 1/well) were then plated into 24-well tissue culture plates on day 5 and incubated for five additional days. To estimate efficiency of differentiation of ES cells into cardiomyocytes, cultures were analysed under an inverted phase-contrast microscope (Olympus, Tokyo, Japan) to examine distinctive beating movements of newly differentiated cardiomyocytes.

Cytotoxicity assay

To study for cytotoxic effects of hyperforin we followed the EST method (10) with some modifications. ES cells and NIH/3T3 fibroblasts in 100 μl culture medium containing appropriate dilution of hyperforin were seeded into 96-well flat-bottomed tissue culture microtitre plates and incubated in a humidified atmosphere with 5% CO_2 at 37 °C. On days 3 and 5, culture medium was removed. Subsequently, 100 μl of the same concentration test substance used on day 0 was added to the microtitre plates. After 24 h, 72 h or 7 days incubation with reagents, cell viability was determined using a CellTiter-Glo luminescent cell viability assay (Promega, Tokyo, Japan). Control cells cultured according to the same procedure, but without hyperforin, were considered to be 100% viable. Cell viability of each drug-treated sample was presented as percentage viability of control cells cultured without hyperforin.

Analysis of apoptosis

Quantification of apoptotic cells was performed using a cell death detection ELISA^{PLUS} (Roche Diagnostics, Tokyo, Japan). This assay allows specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates. Enrichment of mono- and oligonucleosomes in the cytoplasm occurs due to DNA degradation in apoptotic cells before plasma membrane breakdown. After 24 or 72 h incubation with reagents, cells were lysed in lysis buffer (included in the kit) and the assay was performed according to the manufacturer's instructions. Absorbance values were measured at 405 nm using a microplate reader (ARVO; PerkinElmer Japan, Kanagawa, Japan). Apoptotic ratio of cells for each drug-treated sample is presented as fold-change from that of untreated samples. All samples were run five times per assay.

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5-bromo-2′-deoxy-uridine (BrdU) incorporation assay

DNA synthesis levels were determined by measuring BrdU incorporation using the commercial Cell Proliferation ELISA System (Roche Diagnostics). After 24 or 72 h incubation with reagents, cells were incubated for 3 h in BrdU labelling solution containing 10 μM BrdU (included in the kit); the assay was performed according to the manufacturer's instructions. Absorbance values were measured at 405 nm using a microplate reader. All samples were run five times per assay.

Caspase assays

Caspases -3, -8, and -9 are important mediators of apoptosis; caspases -8 and -9 are initiators and caspase-3 is the executioner enzyme (15). Caspase-3/7, -8, and -9 activities were assayed with Caspase-Glo Assays (Promega) according to the manufacturer's respective standard cell-based assay protocol. Luminescence of each sample was measured using a plate-reading luminometer. Comparison of luminescence of a treated sample with that of a control sample enabled relative increase in caspase activity to be determined. All samples were run five times per same assay.

RNA isolation, cDNA synthesis and quantitative RT-polymerase chain reaction

Total RNA was extracted from samples on day 10 of the differentiation assay, and from undifferentiated samples, using the RNeasy RNA extraction kit (Qiagen, Tokyo, Japan). cDNA was synthesized using RNA and PrimeScript II 1st strand cDNA Synthesis Kit (Takara, Shiga, Japan). To analyse relative expression levels of various mRNAs, amount of cDNA was normalized on the basis of signals from ubiquitously expressed GAPDH mRNA. Real-time polymerase chain reaction was carried out using an SYBR Premix Ex Taq II (Takara) and a thermal cycler dice real time system (Takara) according to the manufacturer's standard instructions, to final volume of 25 μl. Primer sequences are summarized in Table 1.

Statistical analysis

Values are expressed as mean ± SEM. Statistical analyses were performed using unpaired Student's *t*-test or two-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test *post-hoc*. Probability values (*P*) of less than 0.05 were considered statistically significant.

Results

Effects of hyperforin on ES cell and fibroblast viability

In both ES cells and NIH/3T3 fibroblasts, hyperforin at high concentrations inhibited cell survival in a dose-dependent manner, after 7 days of hyperforin treatment (Fig. 1). However, there were some differences between

Table 1. PCR primers used in this study for the detection of tissue-specific marker gene expression

Gene symbol	Official full name (NCBI reference sequence)	
	Forward (5′→3′)	Reverse (5′→3′)
POU5F1 (Oct3/4)	POU domain, class 5, transcription factor 1 (NM_013633.3)	
	GGTGGAGGAAGCCGACAAC	TTCGGGCACTTCAGAAACATG
SOX2	SRY-box containing gene 2 (NM_011443.3)	
	AGATGCACAACTCGGAGATCAG	CCGCGGCCGGTATTTATAAT
GATA6	GATA binding protein 6 (NM_010258.3)	
	CGGTCATTACCTGTGCAATG	GCATTTCTACGCCATAAGGTA
TTR	transthyretin (NM_013697)	
	GTCCTCTGATGGTCAAAGTC	TCCAGTTCTACTCTGTACAC
BMP4	bone morphogenetic protein 4 (NM_007554.2)	
	CTGCCGTCGCCATTCACTAT	TGGCATGGTTGGTTGAGTTG
NPPA (ANF)	natriuretic peptide type A (NM_008725.2)	
	CGGTGTCCAACACAGATCTG	TCTCTCAGAGGTGGGTTGAC
NES	nestin (NM_016701.3)	
	TGCATTTCCTTGGGATACCAG	CTTCAGAAAGGCTGTCACAGGAG
GFAP	glial fibrillary acidic protein (NM_001131020.1)	
	TGCCACGCTTCTCCTTGTCT	GCTAGCAAAGCGGTCATTGAG
GAPDH	glyceraldehyde-3-phosphate dehydrogenase (NM_008084.2)	
	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC

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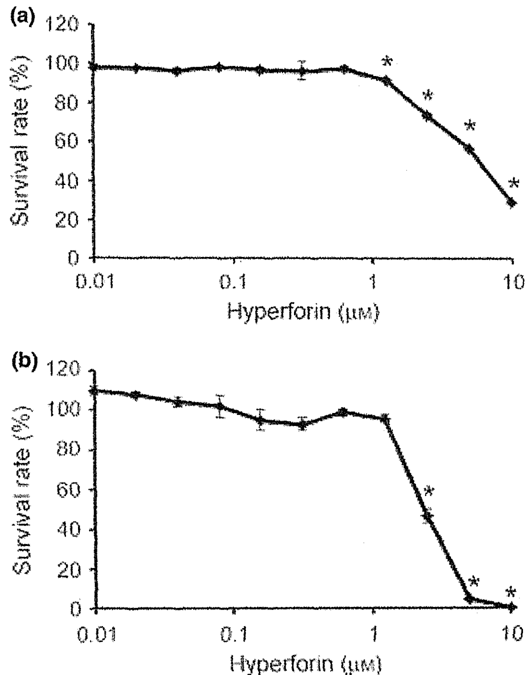

Figure 1. Effect of hyperforin on embryonic stem (ES) cell and fibroblast viability. Employing mouse ES cells (a) and NIH/3T3 cells (b) were treated with hyperforin at indicated concentrations for 7 days. Cell viability was measured by CellTiter-Glo luminescent cell viability assay. Cell cultures exposed to 0 μM drug were considered to be 100% viable. Cell viability of each drug-treated sample was presented as a percentage of viability of cultures treated with 0 μM drug. Data are mean ± SEM of results from at least three independent experiments. $^*P < 0.05$, compared to 0 μM.

ES cells (Fig. 1a) and NIH/3T3 fibroblasts (Fig. 1b) in cytotoxic sensitivity to hyperforin. IC_{50} values (inhibitory concentration of 50% cell viability) were 2.38 and 5.89 μM for NIH/3T3 fibroblasts and ES cells, respectively. Proportions of viable cells after treatment with 10 μM hyperforin were 0.32 ± 0.07 and 29.35 ± 0.34% for NIH/3T3 fibroblasts and ES cells, respectively.

Hyperforin inhibited proliferation of ES cells

To further examine effects of hyperforin on proliferation and viability of ES cells, cells were cultured for 24 or 72 h in the absence or presence of increasing concentrations of hyperforin. ES cell population growth was reduced in samples treated with high concentrations of hyperforin compared to untreated control samples after 24 and 72 h, respectively (Fig. 2a,b). Proportions of viable cells after treatment with 10 μM hyperforin were 37.38 ± 1.42 and 16.46 ± 3.13% after 24 and 72 h. To determine whether hyperforin induced death in ES cells, we measured apoptosis after 24 or 72 h hyperforin treatment. The cell death detection ELISA assay indicated that hyperforin could not induce apoptosis, characterized by DNA fragmentation, in ES cells after 24 h (Fig. 2c) and 72 h (Fig. 2d). In addition, to determine whether hyperforin could inhibit proliferation of ES cells, we conducted a BrdU incorporation assay after 24 or 72 h hyperforin treatment. BrdU incorporation assay showed that uptake of BrdU by ES cells was reduced after exposure to hyperforin (Fig. 2e,f). These results indicate that high concentrations of hyperforin inhibited ES cell proliferation.

Hyperforin induced apoptosis of fibroblasts

To further examine effects of hyperforin on proliferation and viability of NIH/3T3 cells, cells were cultured for 72 h in the absence or presence of increasing concentrations of hyperforin. NIH/3T3 cell proliferation was markedly lower in samples treated with high concentrations of hyperforin compared to untreated control samples (Fig. 3a); proportion of viable cells after treatment with 10 μM hyperforin was 6.52 ± 0.25%. To determine whether hyperforin induced apoptosis in NIH/3T3 cells, we measured it after 72 h hyperforin treatment. The cell death detection ELISA assay indicated that hyperforin induced apoptotic cell death in NIH/3T3 cells (Fig. 3b). In addition, activity of caspase-3/7 was significantly increased at growth-suppressive concentration of hyperforin (Fig. 3c), as were those of caspases -8 (Fig. 3d) and -9 (Fig. 3e).

Furthermore, to determine whether hyperforin could inhibit proliferation of NIH/3T3 cells, we conducted BrdU incorporation assay after 72 h hyperforin treatment. This indicated that uptake of BrdU by NIH/3T3 cells was lower after exposure to hyperforin (Fig. 3f).

Effects of hyperforin on tissue-specific marker gene expression in ES cells

To characterize effects of hyperforin in the ES cell differentiation system, we performed quantitative expression analysis of tissue-specific genes (thus of their products) in samples on day 10 of the differentiation assay. Under our cell culture conditions, ES cells mainly differentiated into cardiomyocytes, although various other cell types were also produced. We demonstrated that expression levels of Oct4 and Sox2 (undifferentiated markers) were markedly lower under differentiating conditions compared to non-differentiating conditions, and that these expression levels were enhanced after treatment with 10 μM hyperforin, although they remained below their initial undifferentiated levels (Fig. 4a,b). For endodermal lineages, expression levels of GATA6 and

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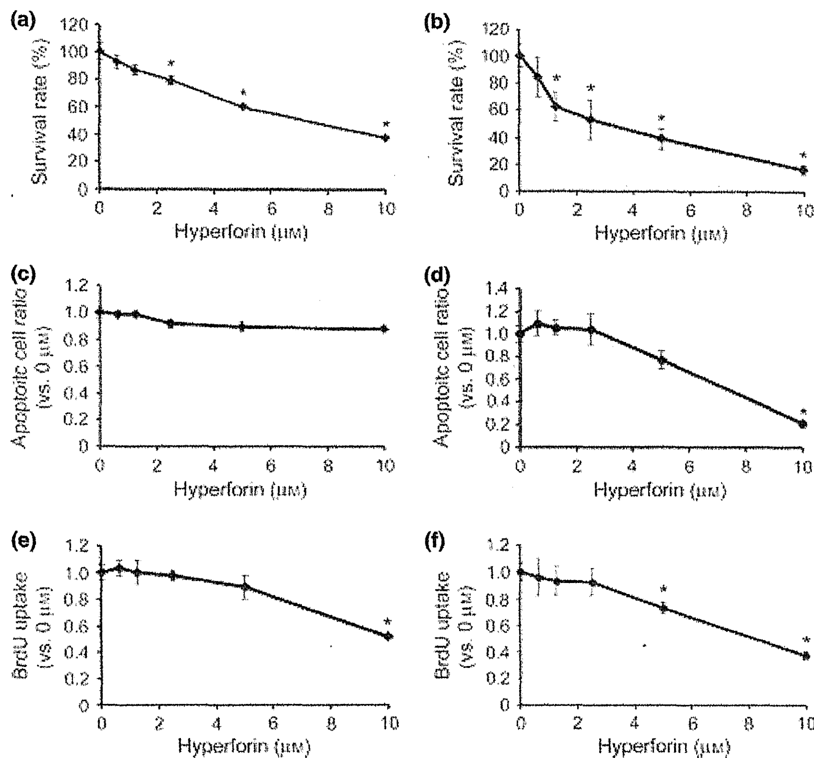

Figure 2. Effects of hyperforin on embryonic stem (ES) cell apoptosis and proliferation. (a, b) Employing mouse ES (mES) cells were treated with hyperforin at indicated concentrations for 24 h (a) or 72 h (b). Cell viability was measured by CellTiter-Glo luminescent cell viability assay. Cell cultures exposed to 0 μM drug were considered to be 100% viable. Cell viability of each drug-treated sample was presented as percentage of that of cultures treated with 0 μM drug. (c, d) mES cells were treated with hyperforin at indicated concentrations for 24 h (c) or 72 h (d). Apoptosis was measured by cell death detection ELISA assay. Apoptotic level in each drug-treated sample was presented as fold-change compared to that in cultures treated with 0 μM drug. (e, f) mES cells were treated with hyperforin at indicated concentrations for 24 h (e) or 72 h (f). Uptake of BrdU was measured by ELISA. BrdU incorporation in each drug-treated sample was presented as fold-change compared to that in cultures treated with 0 μM drug. Data are the mean ± SEM of results from at least three independent experiments. $^{*}P < 0.05$, compared to 0 μM.

TTR were markedly higher under differentiating conditions compared to non-differentiating conditions, and high concentrations of hyperforin reduced these expression levels (Fig. 4c,d). Expression of mesodermal markers BMP4 and ANF were also markedly higher under differentiating conditions compared to non-differentiating conditions, and high concentrations of hyperforin reduced these expression levels (Fig. 4e,f). For ectodermal lineages, expression levels of neuron-specific markers such as nestin and Glial markers such as GFAP were not significantly affected by hyperforin (Fig. 4g,h). Proportion of induced cardiomyocytes was determined based on their autonomous contractile motions which was shown to reduce in a hyperforin concentration-dependent manner (Fig. 4i).

Discussion

In this study, we demonstrated that high concentrations of hyperforin significantly reduced viability of ES cells (an embryonic tissue cell model) and NIH/3T3 fibroblasts (an adult tissue cell model).

Data we obtained from our various analyses suggest that hyperforin at high concentrations inhibits ES cell proliferation. Proliferating somatic cells spend much of their mitotic cycle in the G1 phase, and their progression to S phase is largely controlled by cyclin-dependent kinases (CDK), whose activity is regulated by various cyclins. Although they are expressed in ES cells, in some cases several major CDK-cyclin control complexes, including Cdk4/cyclin D, appear to exhibit little or no regulatory activity. Instead, division of ES cells is driven by the Cdk2/cyclin A/E pathway, constitutively active throughout the cell cycle (16). Given that hyperforin inhibits proliferation of ES cells, it may affect the function of cell cycle regulators such as the Cdk2/cyclin A/E pathway.

On the other hand, it is acknowledged that there are several types of cell death, including apoptosis, necrosis and autophagic cell death. Cells are archetypically known

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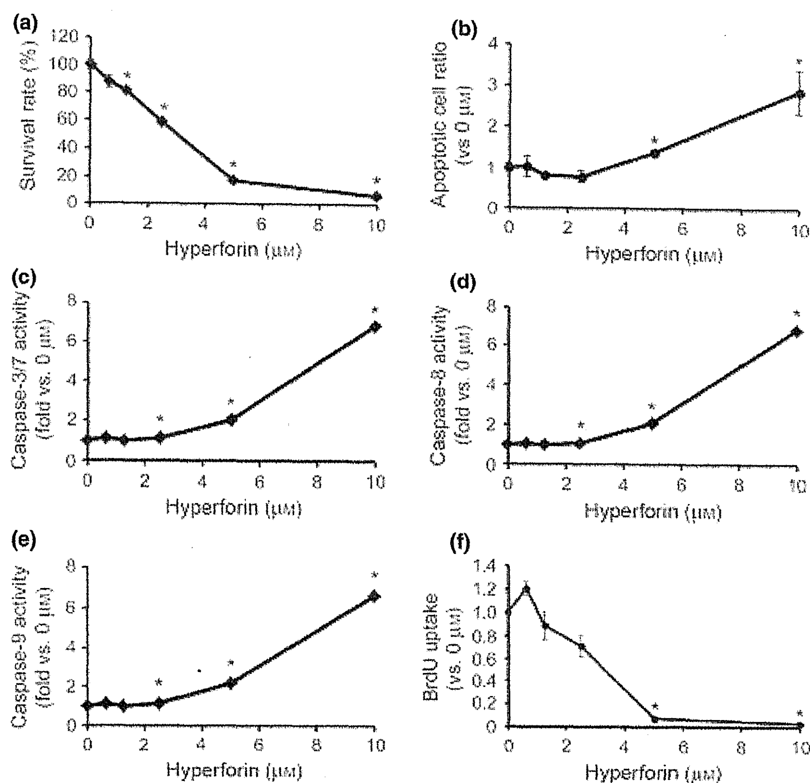

Figure 3. Effects of hyperforin on fibroblast apoptosis. (a) NIH/3T3 cells were treated with hyperforin at indicated concentrations for 72 h. Cell viability was measured by CellTiter-Glo luminescent cell viability assay. Cell cultures exposed to 0 μM drug were considered to be 100% viable. Cell viability of each drug-treated sample was presented as percentage of that of cultures treated with 0 μM drug. (b) NIH/3T3 cells were treated with hyperforin at indicated concentrations for 72 h. Apoptosis was measured by cell death detection ELISA assay. Apoptotic level in each drug-treated sample was presented as fold-change compared to that in cultures treated with 0 μM drug. (c–d) NIH/3T3 cells were treated with hyperforin at indicated concentrations for 72 h. Caspase-3/7 (c), -8 (d) and -9 (e) activities were determined using Caspase-Glo Assays. Data are expressed as fold-increases relative to respective untreated samples (RLU/60 min/μg protein). (f) NIH/3T3 cells were treated with hyperforin at indicated concentrations for 72 h. Uptake of BrdU was measured by ELISA. BrdU incorporation in each drug-treated sample was presented as fold-change compared to that in cultures treated with 0 μM drug. Data are expressed as mean ± SEM of results from at least four independent experiments. *$P < 0.05$, compared to 0 μM.

to disassemble in two morphologically and biologically distinct processes, namely, programmed cell death (PCD) and necrosis. PCD, referring to apoptosis, autophagy and programmed necrosis, is proposed to be death of a cell in any pathological format, when mediated by an intracellular program. These three forms of PCD may jointly decide the fate of cells; apoptosis and programmed necrosis invariably contribute to cell death, whereas autophagy can play either a pro-survival role or a pro-death role (17). Cells undergoing apoptosis show a series of well-characterized physical changes such as plasma membrane blebbing, permeabilization of mitochondrial outer membranes, DNA fragmentation, nuclear disintegration and eventually cell disintegration into apoptotic bodies that are then engulfed and degraded by phagocytes (18). On the other hand, autophagy (macroautophagy) occurs by formation of autophagosomes, double-membraned vesicles that sequester organelles, proteins, or portions of cytoplasm, which then fuse with lysosomes. As a result of this process, sequestered contents are degraded by

Figure 4. Effect of hyperforin on embryonic stem (ES) cell differentiation. Analysis of expression levels for undifferentiated state and tissue-specific differentiation markers. Expression levels of undifferentiated markers Oct3/4 (a) and Sox2 (b), endodermal markers GATA6 (c) and TTR (d), mesodermal markers BMP4 (e) and ANF (f) and ectodermal markers nestin (g) and GFAP (h) were quantified at each concentration of hyperforin with real-time RT-PCR. Each experiment was performed in triplicate. Data are expressed as mean ± SEM of results from at least three independent experiments. *$P < 0.05$, compared to 0 μM. un, undifferentiated ES cell. (e) The frequencies of cardiomyocytes, identified by their distinctive beating movement, derived from ES cells were quantified at each concentration of hyperforin.

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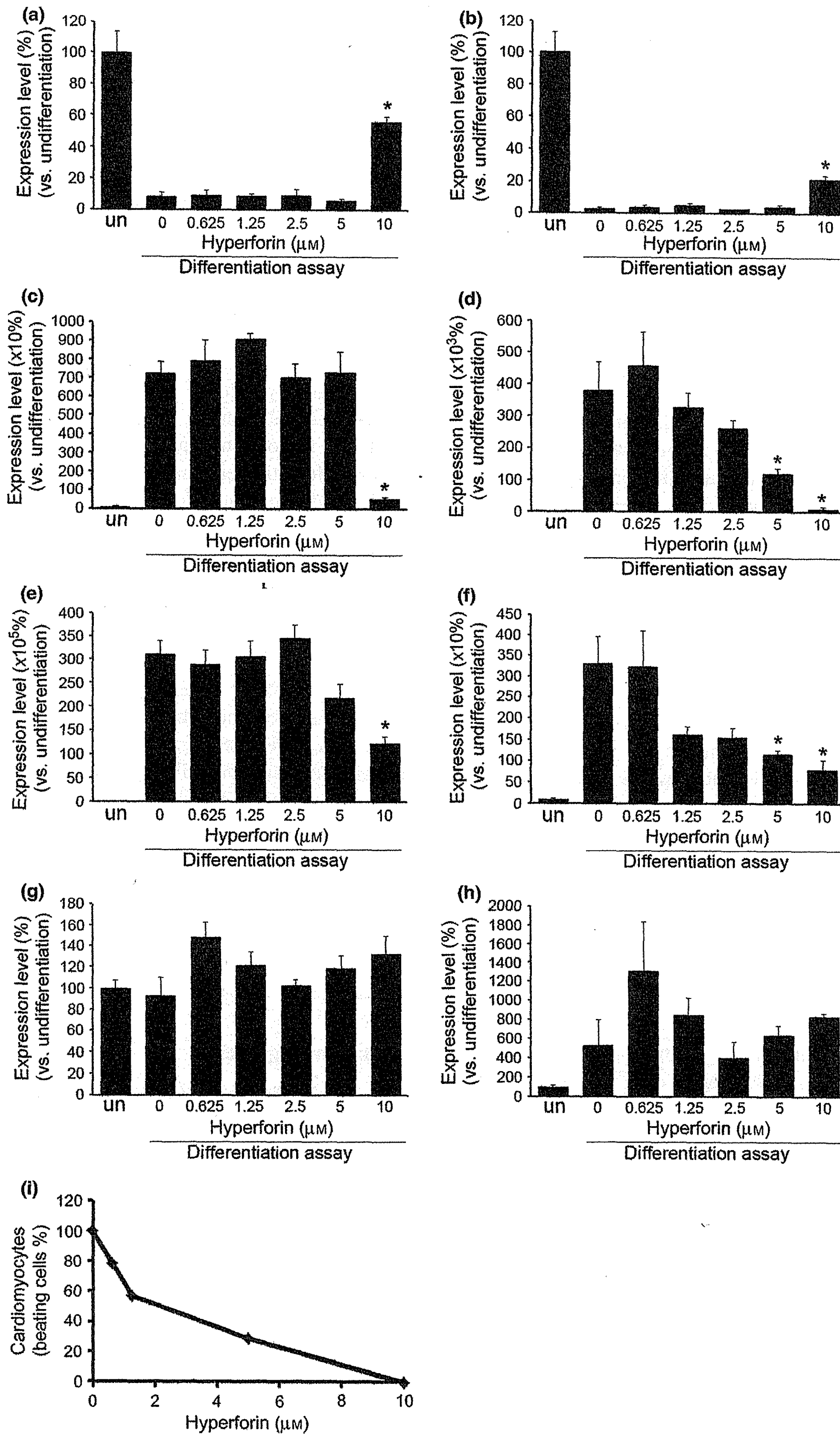

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lysosomal enzymes and recycled for future re-use (19). Autophagy does not involve DNA fragmentation (20). Our results here show that hyperforin did not induce apoptosis in ES cells. It is not known, however, whether other types of cell death such as necrosis or autophagy contribute to ES cell growth inhibition.

The molecular mechanisms that account for the hyperforin-induced cell viability reduction of ES cells remain to be investigated.

Our data also suggest that NIH/3T3 fibroblast cytotoxicity to hyperforin at high concentrations induced apoptosis. During apoptosis, caspases are essential for initiation and execution of cell death in a self-amplifying cascade in response to various stimuli (21). Two major apoptotic pathways have been identified, one extrinsic and one intrinsic. The extrinsic pathway is activated by death receptors, which recruit initiator caspases -2, -8, or -10 through adaptor molecules, whereas intrinsic signals result in activation of caspase-9. Initiator caspases can sequentially cleave and activate effector caspases (caspases -3, -6, and -7), which play an important role in mediating cell destruction (22). Our results show that hyperforin increased mono- and oligonucleosomes and activities of caspases -3/7, -8 and -9 in NIH/3T3 fibroblasts, indicating that it induced not necrosis nor autophagy, but apoptosis of NIH/3T3 fibroblasts, *via* both the intrinsic pathway (as shown by activation of caspase-9) and the extrinsic pathway (as shown by activation of caspase-8), and also led to caspase-3 activation.

Thus, our results suggest that hyperforin affected cytotoxicity in a cell-dependent manner; it inhibited proliferation of ES cells (representing embryonic tissue) and induced apoptosis of fibroblasts (representing adult tissues). Effects and the molecular mechanisms of hyperforin must therefore be examined further, in individual cells and tissues.

It must be noted that concentrations of hyperforin taken into the body through consumption of St. John's Wort are very low. Agrosi *et al.* (23) observed peak plasma hyperforin level of 168.35 ng/ml (about 30 nM) after consumption of a soft gelatin formulation and 84.25 ng/ml (about 15 nM) after consumption of hard gelatin capsules. Vitiello *et al.* (24) were unable to detect any hyperforin in 17 of 97 volunteers who took St. John's Wort. Hyperforin had an IC_{50} value of around 2.4 μM for NIH/3T3 fibroblasts and around 5.9 μM for ES cells as measured for this study; these levels are approximately 80–200 times plasma hyperforin level observed by Agrosi *et al.* These results suggest that hyperforin can be expected to have few embryotoxic effects in general use.

In a previous study, subjects taking St. John's Wort were prospectively identified, followed and compared with a matched group of pregnant women taking other forms of pharmacological therapy for depression, and a third group of healthy women not exposed to any known teratogens. It was demonstrated that levels of major malformations were similar across the three groups: they were 5%, 4% and 0% in St. John's Wort, disease comparator and healthy groups, respectively. These levels are not significantly different from the 3–5% risk expected in the general population. Levels of live birth and prematurity also did not differ among the three groups (25). In this study, using an assay system based on the EST developed in our previous studies (12–14), we have demonstrated that high concentration (10 μM) of hyperforin increased expression level of undifferentiated marker genes and reduced expression level of mesodermal and endodermal marker genes under differentiating conditions. In addition, hyperforin inhibited differentiation of ES cells into cardiomyocytes. We found, however, that undifferentiated marker expression levels were lower in ES cells that had been subjected to 10 μM hyperforin treatment under differentiating conditions than they were in undifferentiated ES cells, while mesodermal and endodermal marker expression levels were higher in ES cells that had been subjected to 10 μM hyperforin treatment under differentiating conditions than they were in undifferentiated ES cells. These results suggest that high concentrations of hyperforin could partially inhibit ES cell differentiation into mesoderm and endoderm lineages, and that ingestion of large amounts of hyperforin could pose embryotoxic and teratogenic risks.

Hyperforin is currently in used in the clinic as a safe and effective antidepressant. Our experimental data indicate that it could be expected to have few embryotoxic and teratogenic effects in general use, although ingestion of large amounts of hyperforin may incur risks.

Acknowledgements

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