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(54) Titre : ESSAI D'AGRESSION OXYDATIVE AU MOYEN DE SUSPENSIONS DE CELLULES
 (54) Title: TEST FOR OXIDATIVE STRESS USING CELL SUSPENSIONS

(57) **Abrégé/Abstract:**

This invention provides means of measuring oxidative response of cells without recourse to preparation of cell culture. The process consists of: 1) preparing suspensions of cells from a living host in isotonic solutions, 2) preparing samples of test materials in isotonic solution containing tagged choline, 3) adding the cells suspension prepared in step 1 to the samples prepared in step 2, 4) incubating the product of step 3 with shaking for 2-90 minutes, 5) extracting and drying the lipid phase from the product of step 4, and 6) measuring the choline that has been incorporated onto PC in the product of step 5. An increase in incorporation of choline into PC in the short term indicates oxidative stress of free radical induced damage. Because the method of the invention using the cell isolates does not require the expense of cell culture with concomitant expense and possibility of cell change, it is particularly useful for clinical evaluation. Furthermore, the ability to use erythrocytes for study of cellular response to oxidative stress makes it possible to test individual responses of patient using part of the blood sample drawn routinely for laboratory use.

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(54) Title: TEST FOR OXIDATIVE STRESS USING CELL SUSPENSIONS		
(57) Abstract This invention provides means of measuring oxidative response of cells without recourse to preparation of cell culture. The process consists of: 1) preparing suspensions of cells from a living host in isotonic solutions, 2) preparing samples of test materials in isotonic solution containing tagged choline, 3) adding the cells suspension prepared in step 1 to the samples prepared in step 2, 4) incubating the product of step 3 with shaking for 2-90 minutes, 5) extracting and drying the lipid phase from the product of step 4, and 6) measuring the choline that has been incorporated onto PC in the product of step 5. An increase in incorporation of choline into PC in the short term indicates oxidative stress of free radical induced damage. Because the method of the invention using the cell isolates does not require the expense of cell culture with concomitant expense and possibility of cell change, it is particularly useful for clinical evaluation. Furthermore, the ability to use erythrocytes for study of cellular response to oxidative stress makes it possible to test individual responses of patient using part of the blood sample drawn routinely for laboratory use.		

APPLICATION FOR LETTERS PATENT

Title: Test for Oxidative Stress Using Cell Suspensions

Field of the Invention:

5 This invention provides a method for evaluation of
cytotoxic effects of oxidative stress on cells while avoiding
the need for growth of the cells in tissue culture. The
methods of the invention make it possible to test agents such
as potential drugs or other bioactive agents, such as pesti-
10 cides, which may be introduced into the environment for
purposes of predicting cytotoxic effects on animal populations.
Using the methods of the invention, it is also possible to
obtain predictive information about cellular response of the
intended individual toward a given bioactive agent.

15 Background of the Invention:

The cells of all mammalian tissues require oxygen for
respiration and oxidative metabolism. Unfortunately, the
cellular reduction of oxygen results in the formation of
reactive oxygen species (ROS) which are cytotoxic.

20 The membranes of all mammalian cells contain a phospholip-
id bilayer in which are imbedded various proteins that regulate
drug transport, signal transduction and cellular metabolism.
Phosphatidylcholine (PC) is the major membrane phospholipid.
The PC contains an abundance of polyunsaturated fatty acids
25 (PUFA) which are excellent free radical traps. Free radicals
generated by cellular monooxygenases, which are imbedded in the
membrane's phospholipid bilayer, produce membrane injury by
interacting with the PUFA's of membrane-associated PC. The
cell will die unless the free radical-induced membrane damage
30 (alterations in PC structure) is rapidly repaired, since
required cellular functions such as chemical transport, signal
transduction and metabolism are disrupted. Therefore, the cell
rapidly activates (by enzyme translocation) PC hydrolysis and
biosynthesis to repair the injured membrane. In the healthy
35 cell, reversible injury is repaired by rapid (5-10 minute) and

significant increases (about 2-3 fold) in cellular PC biosynthesis. This process can be measured by determining the cellular incorporation of labeled choline into PC. However, free radical-induced membrane injury is only reversible as long as the rate of membrane injury is not greater than the cell's rate of membrane repair. If cells are continuously injured for extended periods, the cell's ability to make PC decreases, resulting in irreversible injury and cell death.

The "free radical" theory of cell injury has been proposed for many years to explain how cell death is produced by various conditions such as alcoholic liver disease (ALD), tissue dysfunction associated with aging, traumatic brain injury, drug-induced tissue injury, reperfusion cell damage, irradiation and exposure to UV light. Cellular levels of free radicals increase when cellular content of oxidants and antioxidants increase and decrease, respectively. The resulting injury is known as oxidative stress.

Free radicals are very reactive and can not move far from their site of formation, since they will readily interact with various cellular components such as proteins and phospholipids. The P450-dependent monooxygenases that are imbedded in the phospholipid bilayer of cellular membranes are a major source of reactive oxygen species such as superoxide anion and hydrogen peroxide that are generated during the cellular metabolism of various agents.

It is believed that the interaction of redox active ion, superoxide anion and hydrogen peroxide produce the toxic hydroxyl radical by a Haber-Weiss reaction. Cell injury occurs, in part, when the hydroxyl radical interacts with the polyunsaturated fatty acids (PUFA's) of membrane phospholipids such as PC. Free radical-induced membrane injury is repaired if PC hydrolysis and biosynthesis are rapidly increased. However, cell death occurs if PC metabolism is not increased.

The above theory of cell injury as outlined is reasonable. However, determining the validity of this theory is difficult. The primary problem is that free radicals are very reactive, short-lived chemical entities. Therefore, it is difficult to

measure the cellular level of free radicals and the effect of free radicals on cell functions. One way to overcome this problem is to incubate isolated hepatocellular fractions with labeled bioactive agents such as carbon tetrachloride and bromotrichloromethane (BTM) and thereafter determine the covalent binding of the trichloromethyl radical ($\cdot\text{CCl}_3$) to cellular components such as phospholipids and proteins. The trichloromethyl radical is rapidly bound covalently to the PUFA's of cellular PC. This free radical interaction can not be detected by measuring lipid peroxidation, since hydrogen abstraction has not occurred. Nevertheless, lipid peroxidation is routinely used to assess the reaction of free radicals with PUFA's. As a result, most investigators have concluded that the interaction of free radicals with cellular PC is a late, rather than early, event in the pathogenic sequence of cell death. The instant invention provides a much improved method for measuring the interaction of free radicals with cellular PC.

Previous studies have shown that oxidative stress can be induced and demonstrated in cultured cells by measurement of the incorporation of labeled choline into phosphatidylcholine. The use of cells grown in cell culture presents several problems for the investigator. First, the propagation of the cells outside of the initial, natural host results in changes in the cells. The farther in time and/or generation that the cells of the culture are from the host that supplies the cells, the more likely it is that the cells have undergone changes that alter the oxidative response. Furthermore, the culturing of some cells is often difficult, costly and time-consuming. Finally, it is quite expensive to culture cells from an individual to get a reading of how the individual host cells (as opposed to the cells generated in culture as representative of the species and cell type) will respond to a given bioactive agent.

Previous studies in tissue culture have shown that the initial response to oxidative stress is an increase in cellular phosphatidylcholine (PC) biosynthesis, which represents the

cell's attempt to repair damage. In the long term, however, there is a decrease in PC biosynthesis because the cell's repair function is damaged, eventually causing cell death.

Ferrali, et al (Biochem Pharm., Vol 36, No. 11, pp 1819-1825 (1989)) teaches the reporters could not demonstrate the adverse effects of allyl alcohol and acrylic acid in erythrocytes. They did manage to show deleterious effects on the cells arising from exposure to acrolein. It was suggested that the damage to the cells resulted from the effects of iron delocalization but did not provide definitive results in tests for oxidative stress from allyl alcohol. However, using the methods taught therein, those researchers were unable to show effect on erythrocytes and concluded that the enzyme required for oxidative damage from allyl alcohol was not demonstrated because the cells lacked alcohol dehydrogenase. Hence, their method did not give definitive results in tests for oxidative stress which was known to occur. This problem has been solved using the method of the invention when studying suspensions of erythrocytes.

Summary of the Invention:

This invention provides means of measuring oxidative response of cells without recourse to preparation of cell culture. The process consists of:

- 1) preparing suspensions of cells from a living host in isotonic solutions,
- 2) preparing samples of test materials in isotonic solution containing tagged choline,
- 3) adding the cell suspension prepared in step 1 to the samples prepared in step 2,
- 4) incubating the product of step 3 with shaking for 2-90 minutes,
- 5) extracting and drying the lipid phase from the product of step 4, and
- 6) measuring the choline which has been incorporated into PC in the product of step 5.

The method as exemplified herein for practicing step 6 involves subjecting the product of step 5 to a scintillation counter to

measure choline which has been incorporated into phosphatidylcholine (PC).

During the practice of step 5, the lipid phase may be subjected to repeated washings.

An increase in incorporation of choline into PC in the short term indicates oxidative stress or free radical-induced damage.

This invention also provides a method of evaluating potential oxidative stress and/or cytotoxic response changes induced by at least one agent of interest on cells from a living animal. The method comprises the following steps:

- 1) obtaining a sample containing cells from the animal and, without intervening cell culture, preparing at least two suspensions of cells from the sample in isotonic solutions;
- 2) preparing a control solution containing detectably labelled choline, and adding one of the at least two non-cultured suspensions thereto to form a control suspension;
- 3) preparing at least one test solution each containing detectably labelled choline and the at least one agent of interest, and adding a different one of the at least two non-cultured suspensions to each of the at least one test solution to form at least one test suspension;
- 4) incubating the control and at least one test suspensions for 2-90 minutes under conditions suitable for incorporation of said detectably labelled choline into phosphatidylcholine in a lipid phase of the said animal cells therein;

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- 5) stopping the incubating by adding an organic solvent to each of the control and at least one test suspensions;
- 6) extracting and drying the lipid phase of the cells in each of the stopped control and test suspensions;
- 7) detecting and measuring labelled choline incorporated into each lipid phase, and comparing a level of choline incorporation into phosphatidylcholine measured in the lipid phase of cells in the at least one test suspension to a level of incorporation measured in the control suspension, to identify differences in the levels of labelled choline incorporation in order to evaluate oxidative stress and/or cytotoxic response effects induced by the agent of interest on the cells of the living animal.

Detailed Description of the Invention:

It is the purpose of this invention to make it possible to study oxidative response on cells without resort to tissue culture. Using the processes taught herein, it is possible to obtain valuable information relating to the free radical-induced damage to the cells. It was not previously known that such testing could be done using cell suspensions.

It is particularly interesting that it is possible to use erythrocytes to measure oxidative stress arising from various toxic agents. The methods of the invention make it possible to test the effects of agents on cells of a particular patient to identify individual cellular response. The process is useful for determining the cytotoxic potential of various agents individually and in combination. Additionally, agents may be tested in combination with known toxins to determine whether

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they possess protective properties. When red blood cells are used, it is possible, using the process of the invention, to determine whether or not a particular agent is hemolytic.

The following PC metabolism theory of free radical-induced cell injury is proposed damage arising from exposure to alcohol. ROS(O_2 and H_2O_2) and acetaldehyde are produced by metabolism of ethanol by alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP 2E1). (CYP 2E1 can also metabolize other drugs such as acetaminophen.) Glutathione (GSH) is then depleted by GSH peroxidase and conjugation with acetaldehyde. Hydroxyl radicals are rapidly formed by a Haber-Weiss reaction. Thereafter, hydroxyl radicals produce cell injury by rapidly interacting with the PUFA's of membrane PC. Membrane damage is repaired if PC hydrolysis and biosynthesis are rapidly increased (reversible damage). However, cell death occurs when

the rate of membrane injury exceeds the membrane's repair capacity.

Methodology

5 Preparation of cell suspensions: The following methods exemplify the technology (known in the literature) for isolation of cells.

Rat Hepatocytes:

10 Male sprague Dawley rats (Zivic-Miller) weighing 200 to 350 grams were treated with a 50 mg/kg dose of phenobarbital (ip) for 3 days prior to perfusion. During this 3 day period, the animals were also given water ad libitum containing 0.1% sodium phenobarbital. The animal was anesthetized with ether under a fume hood, then transferred to the surgical table with the abdomen exposed. The belly was shaved and cleansed with 15 70% ethanol and Clinidine. The abdominal skin was removed with scissors and forceps. An incision was made along the base of the abdomen and the renal artery was tied off.

20 After the hepatic vein was cannulated with a 16 gauge catheter, the liver was washed with a balanced salt solution containing EDTA and Tricine. The hepatic artery was cannulated with a 16 gauge catheter. After 5 minutes of reverse flow perfusion was practiced to facilitate the wash cycle. After the blood is washed from the liver, the color of the liver changes from deep red to a brownish color.

25 The liver was then infused with Collagenase solution (112.5 mg/300 ml of Waymouth 752/1 media) bubbled with 5% CO₂. After 5 minutes, reverse flow of Collagenase into the liver was accomplished, followed by reverse to normal flow after 5 minutes. Normal flow was continued until liver tissue was 30 breaking down and ready for collection. Complete liver wash and perfusion should take about 30 minutes.

35 The perfused liver was collected with a strainer and transferred to an Erlenmeyer flask containing 50 ml of original Collagenase solution. The liver was minced using a gyratory water bath at 37 °C and allowed to swirl for no longer than 15 minutes. The solution was then filtered through a sterile gauze into centrifuge tubes and spun in a table top centrifuge

for 5 minutes at 1000 rpm. (70 x g). Aspirate media and suspended cells were placed in Waymouth 752/1 media containing 20% isodensity Percol and were then centrifuged for 4 minutes at 70 x g. Cells were washed three times and resuspended in Waymouth 752/1 media. Samples of 0.5 ml were plated on 35 mm collagen-coated plastic culture dishes containing 0.75 ml of supplemented Waymouth 752/1 media.

The isolated cells were also added to test tubes containing 0.75 ml of Waymouth 752/1 media. Various agents were added and mixtures incubated at 37°C for various periods of time as exemplified below. Incubations were terminated by the addition of 2 ml methanol. This was followed by extraction of lipids.

In an alternate method, rats were sacrificed and the liver removed by dissection. The weight of the liver was determined, and sufficient PBS was added to provide PBS equal to 4 times the liver weight. (A 10 g liver is diluted in 40 ml of PBS.) The liver was minced and homogenized. The homogenate was placed in plastic Sorval tubes, then centrifuged at 3000 rpm for 10 minutes under refrigeration (1000 x g). The supernate was removed and 40 ml PBS added. This may be quickly frozen in glass test tubes in 3 ml aliquots and stored at -70°C.

Preparation of red cell fractions: Rat or human blood was placed in Sorval tubes and, centrifuged at 3000 rpm for 10 minutes under refrigeration. The supernate was removed and the pellet of RBC's resuspended in PBS at 4 times the volume of the supernate. This may be frozen in 2 ml aliquots at -70°C. (Do not freeze 3 ml aliquots as used for hepatocytes, since these will break.)

PC biosynthesis in cultured cells: Aliquots (0.01 ml) of DMSO and water or DMSO and water containing various agents were added to medium with 2-hour monolayers of cells. After the appropriate agent-incubation period, a 0.02 ml aliquot of [³H]-choline (0.2 μCi and 15 pmoles of choline) was added to Waymouth 752/1 (1.25 ml) media. Incubations were stopped after 90 minutes by addition of 2 ml of methanol containing 1% 1N HCl. Cells were removed from the culture dishes by scraping with TEFLON™-coated spatula and placed in glass test tubes.

(Lamb, et al., Hepatology 19: 174 (1994))

PC extraction procedure: Two ml of chloroform and 0.5 ml of water were added to each sample. Tubes were vortexed and centrifuged for 5 minutes to clear the upper and lower phases. The upper phase was aspirated and the lower phase washed by adding 2 ml of wash solution containing 50% methanol, 45% water and 5% 7.4% KCl. The samples were vortexed and centrifuged 5 minutes to clear the upper and lower phases. The upper phase was aspirated and the lower phase was washed again in 2 ml wash solution. After the phases were cleared by centrifugation, the lower phase was carefully removed, placed in a glass test tube, dried, solubilized in scintillation fluid and counted in a Beckman beta counter. Agent-induced alterations in the incorporation of labeled choline into PC was used as a measure of changes in cellular PC biosynthesis.

PC biosynthesis in isolated cells: Aliquots (0.75 ml) of Waymouth 752/1 media were placed in glass test tubes. Aliquots (0.01) of DMSO and water or DMSO and water containing various test agents were added to the tubes along with 0.02 ml [³H]-choline [0.2 μ Ci and 15 pmoles choline]. Incubations were started by adding 0.5 ml aliquotes of cells suspended in Waymouth 752/1 media to the test tubes and placing the tubes in a shaking water bath at 37°C. Incubations were stopped after the appropriate incubation period (2-90 minutes) by addition of 2 ml of methanol containing 1% 1N HCl. Labeled PC was extracted by methods described above for cultured cells. Agent-induced increases in the incorporation of labeled choline into PC were used to measure increases in cellular PC biosynthesis.

Waymouth 752/1 amino acids (without valine) (A.A. Mix)

<u>Amino Acid</u>	<u>gm/50 liters</u>
L-Aspartic Acid	3.0 gm
L-Cystine	0.75 gm
L-Glutamic Acid	7.5 gm
Glycine	2.5 gm
L-Isoleucine	1.25 gm
L-Leucine	2.5 gm

	L-Proline	2.5 gm
	L-Threonine	3.75 gm
	L-Tyrosine	2.0 gm
	Cysteine	3.05 gm
5	L-Histidine	6.4 gm
	L-Lysine	12.0 gm
	L-Tryptophan	2.0 gm
	L-Methionine	2.5 gm
	L-Phenylalanine	2.5 gm
10	L-Ornithine	1.0 gm

The amino acids were mixed well with mortar and pestle. Mixture was stored at room temperature in a dark bottle.

Example 1:

Alteration in PC biosynthesis of human liver cells incubated 90 minutes with 100 mM ethanol (ETOH) or 1 mM acetaminophen \pm 1 mM 4-methylpyrazole was studied in accord with the methodology disclosed above. It was found that ethanol and acetaminophen significantly ($p < 0.05$) increase cellular PC biosynthesis whereas 4-methylpyrazole significantly decreases the ethanol and acetaminophen-induced increases in cellular PC biosynthesis.

Example 2:

Alteration in PC biosynthesis of rat liver cells incubated 90 minutes with 100 mM ethanol or 1 mM acetaminophen \pm 4-methylpyrazole was studied. Ethanol and acetaminophen significantly ($p < 0.05$) increased cellular PC biosynthesis whereas 4-methylpyrazole significantly reduced the ethanol-induced and acetaminophen-induced increases in cellular biosynthesis.

Example 3:

Primary cultures of adult rat hepatocytes were incubated for 72 hours with 100 mM ethanol (ETOH), 1 mM acetaminophen (APAP), or both. A significant decrease was seen in cellular PC biosynthesis potentiated by PUFA (20:4) and reduced by saturated fat (16:0), 4-methylpyrazole (4-MP), superoxide dismutase (SOD), catalase (CAT), deferoxamine (DEF) and vitamin E succinate (VES). These results suggest that ethanol and

acetaminophen do not produce cell injury until they are metabolically activated by P450 monooxygenases (Note, also, inhibition of injury by 4-methylpyrazole). See Table 1.

Table 1

5 Effect of incubating (72 h) cultured hepatocytes with 100 mM ethanol (ETOH), 1 mM acetaminophen (APAP), 0.5 mM 4, methylpyrazole (4-MP), 200 units of superoxide dismutase (SOD), 800 units of catalase (CAT), 2 mM deferoxamine (DEF), 0.025 mM arachidonate (20:4) and 0.25 mM palmitate (16:0) or combina-
10 tions of these agents on cellular PC biosynthesis.

	Additions	PC biosynthesis	Additions	PC biosynthesis
	Control	101 ± 6	Control	101 ± 6
	ETOH	39 ± 2*	APAP	42 ± 2*
	ETOH + APAP	15 ± 1*		
15	ETOH + 16:0	95 ± 8#	APAP	91 ± 9@
	ETOH + 20:4	18 ± 2#	APAP + 20:4	21 ± 2@
	ETOH + 4-MP	98 ± 8#	APAP + 4-MP	95 ± 7@
	ETOH + SOD	85 ± 8#	APAP + SOD	105 ± 10@
	ETOH + CAT	78 ± 4#	APAP + CAT	97 ± 4@
20	ETOH + DEF	107 ± 7#	APAP + DAF	94 ± 6@
	ETOH + VES	107 ± 10#	APAP + FES	87 ± 11@

25 All data is expressed as a percent of control of 6-9 experimental values. All symbols indicate that values are significantly different ($p \leq 0.05$) from control (*), ETOH(#) or APAP (@) exposed cells.

30 Monooxygenases produce superoxide anion (inhibition by SOD) and hydrogen peroxide (inhibition by catalase) which interact with iron (inhibition by deferoxamine) to produce the toxic hydroxyl radical (inhibition by vitamin E succinate) by a Haber-Weiss reaction. These results suggest that the cytotoxic effects of ethanol and acetaminophen on cultured
35 liver cells in vitro are due in part to the interaction of the toxic hydroxyl radical with the PUFA of membrane PC [potentiation by unsaturated fat (20:4) and inhibition by saturated fat (16:0)]. All of these results suggest that free radicals produce alterations in cellular PC biosynthesis. However,
40 short-term free radical exposure produces increases in cellular PC biosynthesis whereas long-term free radical exposure (Table

1) reduces PC biosynthesis. Understanding how long-term free radical exposure disrupts membrane repair processes is fundamentally important in treating and preventing various disorders that are a result of oxidative stress such as aging, alcoholic liver disease, and drug induced tissue injury.

The use of cell suspensions for evaluation of oxidative stress has many applications. For example, in testing drugs or active agents which will be introduced into the environment, the invention can be used to determine if cells from aged or ill mammals respond differently to drug-induced oxidative stress than cells from young mammals. The methods of the invention are also useful for testing effect on cells of interactive cumulative effects of drug combinations. It is also possible, using methods of the invention, to expose freshly drawn red blood cells (RBC's) of patients who may have been exposed to undetermined drugs or toxins to other candidate active agents that might be used for treatment to determine possible effect of giving the particular candidate agents.

Example 4:

Comparison of response of cultured liver cells from older rats (20 months) with those of cultured cells from younger rats (3 months) when exposed to agents such as ethanol and acetaminophen in the manner described in Example 1 showed interesting results. Cells from young, but not from old rats, exhibit the usual ethanol- and acetaminophen-induced increase in PC biosynthesis after short-term agent exposure. However, in the cells from older rats, short-term response resembled long-term response found in cells from younger rats. Hence, it appeared the cells from the older rats progress quickly or immediately to the irreversible damage effects, essentially by-passing the reparative process indicated by increase in PC biosynthesis. (See Table 2.)

Table 2

Alterations in PC biosynthesis of cultured rat liver cells isolated from 3 and 20 month-old male rats incubated 24 hours with 1 mM acetaminophen (APAP) and 100 mM ethanol (ETOH).

Additions		Percent of control + SEM	
		3 Month	20 Month
10	None	107 ± 7	100 ± 3
	ETOH	147 ± 13*	59 ± 1*#
	APAP	137 ± 11*	38 ± 2*#

All data is expressed as a mean ± SEM of nine experimental values. Similar results have been obtained in cells isolated from at least three different 3 and 20 month old rats. Significance from control (*) and cells isolated from 3 month old rats (#) is $p \leq 0.05$.

The results suggest that drug-induced oxidative stress is more cytotoxic in older cells.

The data also would indicate that agents which can increase the cell's capacity to prevent the cytotoxic effects of oxidative stress may be beneficial in preventing cell dysfunction associated with aging. This conclusion was supported by the following example.

Example 5:

Alterations in PC biosynthesis were studied using cultured liver cells incubated 24 and 72 hours with and without 50 μ M vitamin E phosphate (VEP). The cultured hepatocytes displayed incubation time-dependent decreases in PC biosynthesis unless vitamin E phosphate was added to the cell medium.

Example 6:

Cells were isolated from brain homogenates according to the methods described in the methodology section under "PC biosynthesis in isolated cells". Cell fractions (1,000 x g) were incubated 30 minutes with 1 mM FeSO_4 , 100 mM ethanol, 1 mM bromotrichloromethane (BTM), 1 mM Δ -9-tetrahydrocannabinol (THC) and 1 mM acetaminophen (APAP). Cells showed increase in short-term PC biosynthesis consistent with the reversible

injury phase response. (See Table 3.)

Table 3

Agent-induced alterations in PC biosynthesis of 100 x g brain cell fractions:

5	Agent	Percent control of + SEM
	Control	101 ± 4
	1 mM FeSO ₄	226 ± 8
	100 mM ethanol	173 ± 8
	1mM BTM	194 ± 25
10	1 mM THC	293 ± 21
	1 mM APAP	212 ± 7

All values are significantly increased (p<0.01) above controls.

15 **Example 7:**

The influence of agents ± 4-MP on cultured astrocyte PC biosynthesis was studied. Table 4 below gives the data. The agents used were 100 mM ethanol, 1 mM 4-methylpyrazole (4-MP), 1 mM tetrahydrocannabinol (THC) and combinations of agents.

20 **Table 4**

20	Agent	Percent control of + SEM
	Control	102 ± 8
	100 mM ethanol	199 ± 14
	ethanol + 4-MP	122 ± 5
25	1 mM APAP	159 ± 15
	APAP + 4-MP	118 ± 5
	APAP + ethanol	241 ± 23
	APAP + ethanol + 4-MP	145 ± 8
	THC	201 ± 11
30	THC + 4-MP	148 ± 9
	THC + ethanol	234 ± 14
	THC + ethanol + 4-MP	131 ± 5

35 All values without 4-MP are significantly increased (p<0.05) above controls. 4-MP significantly reduced (p <0.05) the agent-induced increase in PC biosynthesis.

Example 8:

40 Rat red blood cells and human red blood cells (RBC) were tested in accord with the methods of Example 4 using various agents to evaluate short-term response to oxidative stress. Alterations in cellular function in rat RBC's after 10 minutes incubation are shown in Table 5 with results using human RBC's after 30 minutes incubation shown in Table 6. The agents used

included 100 mM ethanol (ETOH), 1 mM acetaminophen (APAP), 1 mM tetrahydrocannabinol (THC) 10 mM allyl alcohol, 1 mM cocaine, and 1 mM bromotrichloromethane (BTM) all with and without 4-methylpyrazole (4-MP).

5

Table 5:**Influence of agents on the rat RBC PC biosynthesis**

Agent	PC biosynthesis % of Control \pm SEM
Control	100 \pm 1
100 mM ethanol	374 \pm 19
1 mM APAP	655 \pm 26
1 mM THC	1142 \pm 65
1 mM cocaine	458 \pm 7
1 mM BTM	533 \pm 29
15 10 mM allyl alcohol	1315 \pm 99
1 mM iron	919 \pm 73

Significance from control is $p < 0.05$ for all values.

Table 6:

20

Drug-induced changes in PC biosynthesis of human RBC's

Agent	PC biosynthesis percent of control \pm SEM
Control	100 \pm 1
100 mM ethanol	222 \pm 9
ethanol + 4-MP	94 \pm 5
25 1 mM APAP	512 \pm 27
APAP + 4-MP	143 \pm 13
1 mM THC	433 \pm 30
THC + 4-MP	129 \pm 11
10 mM allyl alcohol	377 \pm 3
30 allyl alcohol + 4-MP	131 \pm 10
1 mM cocaine	292 \pm 10
cocaine + 4-MP	82 \pm 9
BTM	728 \pm 3
35 BTM + 4-MP	272 \pm 22

Significance from control is $p < 0.05$ for all values without 4-MP. 4-MP significantly ($p < 0.05$) reduces all drug-induced increases in PC biosynthesis.

Example 9:

40 Cells isolated from rat lung, spleen, kidney, brain, intestine and blood were incubated 30 minutes with 1 mM acetaminophen (APAP). It was found that 1 mM APAP significantly ($p < 0.05$) increases PC biosynthesis in all cells. (See table 7.)

Table 7:**Drug-induced changes in PC biosynthesis in various cells**

	Tissue	PC biosynthesis percent of control \pm SEM
	control	102 \pm 7
5	lung	471 \pm 38
	spleen	183 \pm 20
	kidney	181 \pm 11
	brain	172 \pm 14
	intestine	287 \pm 13
10	erythrocytes	236 \pm 13

The short term (reversible injury phase) could be shown at very early stages using the fresh cell isolates. Because the method of the invention using the cell isolates does not require the expense of cell culture with concomitant possibility of cell change, it is particularly useful for clinical evaluation. Furthermore, the ability to use erythrocytes for study of cellular response to oxidative stress makes it possible to test individual responses of patient cells using part of the blood sample drawn routinely for laboratory use.

The methods of the invention are also useful for evaluation of oxidative stress-induced injury to cells from UV light, chemotherapy and irradiation. Previously, there was no technique available which could easily detect the cytotoxic effect of these stress-inducing agents and provide means of ongoing monitoring of patients' cellular response. Using methods of the invention, it is possible to test effect of cytotoxic agents and irradiation on the blood cells to determine effect of such agents on the individual's cells. As the patient's cells are exposed to these influences during therapy, it is possible to evaluate how damaging the effect of a given agent has been on normal cells.

Using the testing methods of the invention, it is possible to evaluate the individual response to environmental radiation. This is particularly valuable for persons whose work environment results in exposure to radiation. The effects of radiation on RBC's were studied using the methods of the invention. In that study, human RBC PC biosynthesis showed dose-dependent response to exposure to radiation, with about 500% increase in response at radiation dose of 10.0 rads.

5 It is also possible to obtain cells from individuals by biopsy or other means such as washings from the gastrointestinal and respiratory passages or from scrapings to predict cellular responses of individuals to candidate drugs or other treatment modalities.

The methods of the invention may also be used to determine whether administration of a particular drug to a patient whose history regarding pharmaceutical intervention is unknown would result in cell injury due to oxidative stress.

10 In addition to testing effects on mammals, the process of testing disclosed herein may also be used for testing effects of agents on birds, fish and mammal. Such testing is particularly valuable in study of agents that will be introduced into the animal's environment.

What I claim is:

- 5 1. A method of evaluating potential oxidative stress and/or cytotoxic response changes induced by at least one agent of interest on cells from a living animal, said method comprising the steps of:
- 10 1) obtaining a sample containing cells from said animal and, without intervening cell culture, preparing at least two suspensions of said cells from said sample in isotonic solutions;
- 15 2) preparing a control solution containing detectably labelled choline, and adding one of said at least two non-cultured suspensions thereto to form a control suspension;
- 20 3) preparing at least one test solution each containing detectably labelled choline and said at least one agent of interest, and adding a different one of said at least two non-cultured suspensions to each of said at least one test solution to form at least one test suspension;
- 25 4) incubating said control and at least one test suspensions for 2-90 minutes under conditions suitable for incorporation of said detectably labelled choline into phosphatidylcholine in a lipid phase of the said animal cells therein;
- 30 5) stopping said incubating by adding an organic solvent to each of the control and at least one test suspensions;
- 6) extracting and drying the lipid phase of the cells in each of said stopped control and test suspensions;

- 5 7) detecting and measuring labelled choline
incorporated into each lipid phase, and comparing a
level of choline incorporation into
10 phosphatidylcholine measured in the lipid phase of
cells in said at least one test suspension to a
level of incorporation measured in said control
suspension, to identify differences in the levels of
labelled choline incorporation in order to evaluate
oxidative stress and/or cytotoxic response effects
induced by said agent of interest on the cells of
15 said living animal.
2. The method of claim 1, wherein the animal is a patient.
3. The method of claim 1, wherein the sample is blood.
4. The method of claim 3, wherein the cells are
erythrocytes.
- 20 5. The method of claim 1, wherein the sample containing
cells is a washing from gastrointestinal or respiratory
passages.
6. The method of claim 1, wherein the sample containing
cells is obtained by scraping.
- 25 7. The method of claim 1, wherein, in step 6, the lipid
phase, after extraction, is washed before drying.
8. The method of claim 7, wherein the lipid phase is
washed at least 3 times before drying.
9. The method of claim 1, wherein the at least one agent
30 of interest in step 3 is an agent suspected of
producing red blood cell lysis in patients with
glucose-6-phosphate dehydrogenase deficiency.
10. The method of claim 2, wherein the patient is in a

5 clinical setting.

11. The method of claim 1, wherein, in step 3, more than one agent of interest are added to said at least one test suspension.

10 12. The method of claim 1, wherein, in step 3, a single agent of interest is added to said at least one test suspension.