Oxidative Stress Monitoring Using In Vitro Systems: Tools and Findings

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7.1 Introduction

Oxidative stress is well-known phenomenon, caused by a shift in the delicate balance between radical generation and scavenging of radical capacity in cells. Reactive oxygen species (ROS) primarily composed of superoxide radicals, hydroxyl radicals, etc. In principle, every molecule including oxygen is known as an oxidant or oxidizing agent if it is capable of accepting electrons (Prior and Cao 1999), and the process of electron loss is known as oxidation. In biology, the process of oxidation is always accompanied by reduction and such reactions are called as redox reactions. Redox reactions are basis for numerous biochemical pathways including biosynthesis and regulation of metabolism. While oxidant and reductant are chemical terms, in biological context these are often known as pro-oxidant and antioxidant, respectively (Kohen and Nyska 2002). Pro-oxidant includes several radical and nonradical species (Halliwell 2006).

Understanding the phenomenon of radical scavenging in real time is challenging primarily due to their transient life and non-availability of highly specific probes for various reactive species. Furthermore, in case of plant-based antioxidant, the separation and purification is costly as well as inefficient due to the complexity of composition. This complexity of studying the effect of plant-based antioxidants is further complicated by synergy of actions that exist between various antioxidants.

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P.K. Maurya, P. Chandra (eds.), *Oxidative Stress: Diagnostic Methods and Applications in Medical Science*, DOI 10.1007/978-981-10-4711-4_7

Another challenge in the conventional antioxidant research is the lack reliable and highly specific measurement of antioxidant capacity different biological samples and food products. A large number of scientific evidences and a number of critical reviews have also been published, yet, opinions vary considerably. Moreover, considerable debate about the criteria for the best method is still on. The fact that most of the antioxidant tests are performed in test tubes but not in the live biological milieu further adds to this debate. More than one type of assays for similar parameter and non-availability of indexing the values and their coordinated integration leaves the researches confused about the antioxidant power. Despite the non-consensus on the best and most suitable method for determination of net oxidative stress and gross antioxidant status, the usage and applicability of these assays are massive and provide a huge amount of data which has pushed several drugs into clinical trials, and it is therefore very important to understand the methodology, pros and cons of existing methods and their suitability depending on cost, instrumentation sample type. This chapter outlines the key methods which are currently in use for the qualitative or quantitative estimation of reactive oxygen species, antioxidant defence and oxidative stress in cell culture systems and some of the animal samples such as plasma, which may be analysed in vitro.

7.2 Various Assays for in Vitro Analysis of Oxidative Stress

There are several means of estimating the reactive oxygen species (ROS) in the cellular systems. Usually in vitro systems are the first line of experimentation regimes for any scientific hypothesis, and therefore cell culture and simulated oxidative stress are usually followed by various assays to evaluate the oxidative stress. A number of experimental variants are known for the determination of reactive oxygen species; some of them provide a direct quantitative measure such as biochemical methods. Other methods may be semiquantitative, but either provide a direct visual estimate (such as microscopy) or semiquantitative estimation of ROS in the intracellular compartments (such as flow cytometry). More precise methods such as electron spin resonance methods rely on different principles and therefore can help us to infer transient changes in the ROS especially the rapid ROS burst and its surge. We may broadly categorize the methods for determination of oxidative species primarily on the basis of instrumentation and type of principle. Majority of methods for the ROS estimation are based on spectrometric methods which include UV-Vis spectrophotometry and an extension luminometry and fluorimetry. Spectrometric methods are simple less time-consuming and cost-effective (Fig. 7.1).

7.2.1 Biochemical Assays (Spectrometry Based)

Biochemical assays primarily include those assays in which the reactive oxygen species or their derivatives are estimated using some chemical reaction which result in the formation of coloured complex or noncoloured complex showing high absorbance in ultraviolet radiation or luminescence or fluorescence which may be measured using luminometer or fluorimeter. We will now discuss some of the common methods used

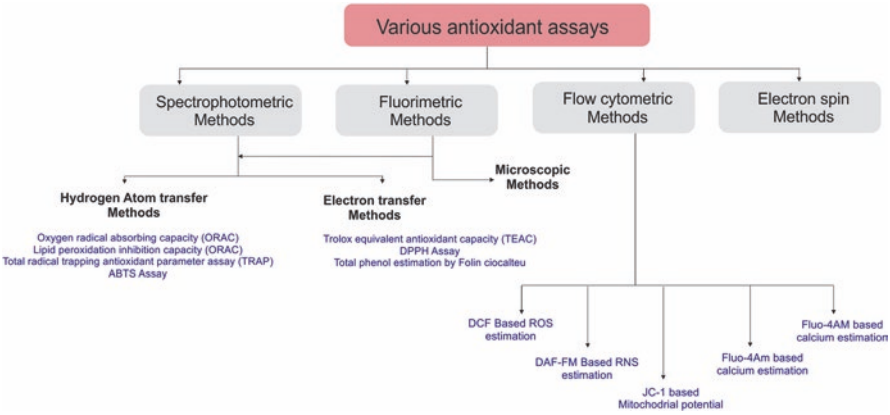

Fig. 7.1 Various methods (assays) for the analysis of antioxidants

for estimation of free radical species and their pros and cons. All the spectrometry-based methods may be broadly grouped into three main categories, hydrogen atom transfer methods (HATMs) and electron transfer methods (ETMs). The third category includes other mechanisms such as peroxidation and other chemical events. The following is the detailed description of commonly used spectrometry-based methods.

7.2.1.1 Hydrogen Atom Transfer Methods

These methods are used for the measurement of the antioxidant capacity or ability to scavenge ROS (e.g. peroxyl radical, ROO•) by the loss of hydrogen ions as shown in equations 1 and 2. ROO• are generally chosen as 64, the reactive species in these assays because of their higher biological relevance and longer half-life (compared to hydroxyl, •OH and superoxide anion radicals, O2 •−). The hydrogen atom transfer (HAT) reaction mechanism, involving the transfer of hydrogen radical/atom (H•) of antioxidants to a peroxyl radical (ROO•) to give more stable free radicals (A• and ArO•), is represented below with the help of chemical equations:

$$\mathrm{ROO}\bullet + \mathrm{AH} \rightarrow \mathrm{ROOH} + \mathrm{A}\bullet \tag{7.1}$$

$$\mathrm{ROO}\bullet + \mathrm{Ar-OH} \rightarrow \mathrm{ROOH} + \mathrm{ArO}\bullet 71 \tag{7.2}$$

$$2\mathrm{ROO}72\bullet \rightarrow \text{Nonradical Products} \tag{7.3}$$

where the radical of the antioxidant (A•) and aryloxyl radical (ArO•) are usually stabilized by resonance. A potent phenolic antioxidant (Ar-OH) need to react faster than the target to be protected with the oxidant (ROS), and A • must be rapidly converted to less reactive species (Apak et al. 2016). Some of the commonly used methods based on HAT are as follows:

A. Oxygen radical absorbing capacity (ORAC) assay

The ORAC assay is one of the commonly used antioxidant assays that involves the inhibition of oxidation of β-phycoerythrin by ROS. Trolox is well-known

reference antioxidant that is used as internal control in the assays. Among several interfering agents, proteins are predominant and reactive groups must be protected during the reaction, due to protein interference especially in plasma. Different radicals have different lag time and therefore different results are expected for the different radicals. Therefore, care must be taken in ORAC assay, when conducted in plasma. As a disadvantage, ORAC cannot be used to determine lipophilic antioxidants but remains limited to the measurement of hydrophilic molecules.

B. Lipid peroxidation inhibition capacity (LPIC) assay

LPIC is an assay that was developed by Zhang et al. in 2006 for assessment of in vitro antioxidant. The lipid peroxidation inhibition capacity (LPIC) method is based on the measurement of both hydrophobic and hydrophilic antioxidants. A lipophilic fluorescent probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid is incorporated in the membrane (Zhang et al. 2006) generating a significance on radical generation which may be further estimated using fluorimetry or flow cytometry.

C. Total radical-trapping antioxidant parameter (TRAP) assay

Total radical-trapping antioxidant parameter or TRAP assay was developed by Wayner et al. It has become the widely used assay to determine total antioxidant activity in biological samples. It is based on the principle of estimating consumption of oxygen under the controlled oxidation of lipids (Wayner et al. 1985). The TRAP results are expressed as the millimolar of peroxyl radicals trapped in plasma. Furthermore, the change in the rate of peroxidation caused by AAPH (2′-azobis (2-amidinopropane) hydrochloride) is monitored indirectly via loss of R-phycoerythrin (R-PE) fluorescence. The lag phase induced by plasma in TRAP assay is same as compared to the lag phase induced by Trolox – a reference antioxidant (Antolovich et al. 2002).

D. ABTS assay

2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS assay was developed by Miller et al. They described an alternative technique for the measurement of total antioxidant capacity (TAC) using colorimetry. The ABTS assay is based on the principle of incubation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS]-(key reagent of the assay) with peroxidase followed by the formation of a relatively stable radical cation, $ABTS^{+}$. Often the ferryl myoglobin is used to facilitate this chemical transition. ABTS+ then forms a relatively stable blue-green colour complex; the absorbance of this coloured solution can be measured at 600 nm using colorimetry or spectrophotometry. Antioxidants present in the fluid samples are known to suppress the formation of coloured complex and therefore remain potential interfering agents (Miller et al. 1993).

7.2.1.2 Electron Transfer Methods (ETMs)

Unlike aforesaid hydrogen atom transfer methods, the electron transfer methods are based on the antioxidant action simulated with a suitable redox potential probe, namely, the antioxidants react with a fluorescent or coloured probe (oxidizing agent) instead of peroxyl radicals. Spectrophotometric ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced. The degree of colour change (either an increase or decrease of absorbance of the probe at a given wavelength) is correlated to the concentration of antioxidants in the sample. The following are some of the commonly used ET-based antioxidant assays:

A. *TEAC* assay

Trolox equivalent antioxidant capacity (TEAC) assay is used to determine the ability of molecules to scavenge the stabilized free radical of 2,2′-azinobis-(3-ethylbenzothiozoline-6-sulphonic acid). Trolox (a hydrophilic analogue of vitamin E) is used as an internal reference for comparing the antioxidant capacity. This technique is not widely applied due to limited scope for the biological samples that can be used. This limits the comparison of the results across various platforms.

B. FRAP assay

Ferric reducing antioxidant power (*FRAP*) method was developed by Antolovich. The principle of this method is reduction of Fe^{3+} complex of tripyridyltriazine Fe $(TPTZ)^{3+}$, a type of a ferroin analogue, which turns into blue-coloured Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants in acidic condition (Antolovich et al. 2002). The blue colour thus obtained is measured at 593 nm using spectrophotometry and used for the determination of Fe^{2+} equivalents which corresponds to the antioxidant standard used in the assay. Sanchez et al. have reviewed the method and report it as simple and rapid method for manual as well as automated procedures (Sanchez-Moreno et al. 2000).

C. DPPH assay

An organic compound 2,2-diphenyl-1-picrylhydrazyl or DPPH is the key component of this assay which is capable of trapping the radicals present in the reaction and forming a coloured compound. The coloured complexes thus formed show an absorption at 517 nm which can be evaluated using spectrophotometry. The antioxidant activity is determined by measuring the decrease in the absorbance (Brand-Williams et al. 1995). Studies have shown that quantity of antioxidant necessary to decrease the 50% of the initial DPPH concentration tEC50 was later used to define a new parameter in the antioxidant research known as antiradical efficiency.

D. Total phenols estimation by Folin-Ciocalteu

Phenolic compounds are the commercially important secondary metabolites often extracted from the plants; some of the common examples of phenolic include

phenolic acids, flavonoids, tannins, lignin, etc. Some of these phenolic compounds are known to be antioxidants and confer UV protective abilities. Determination of the phenolic content in the phytoextracts is also one of the primary assays towards the measurement of total antioxidant capacity. One of the most commonly used methods involves the reaction with Folin-Ciocalteu reagent, which can react with the phenolic group and can produce a coloured complex showing maximum absorbance at 650 nm. The method and various studies based on this method have been recently reviewed by Andressa et al. (Blainski et al. 2013). One of the disadvantages of this method is the interference with the proteins containing high-content or aromatic amino acids, which will also react with the aforesaid reagent.

7.2.1.3 Other Related Spectrometric Methods

A. *Dye-based fluorometric assay in cell lysate*

There are a number of spectrophotometric methods which are based on fluorescent probes which are often used to determine the ROS in cell lysate or tissues homogenates. The degree of fluorescence in the sample is directly proportional to the amount of ROS; however, the absolute quantification cannot be done using these methods, and only relative quantification is obtained. Therefore the amount of ROS in samples is usually represented as fold change or arbitrary units. The most commonly used fluorescent probe is DCFH-DA or dichlorofluorescein diacetate, which is cleaved by intracellular esterases, and then cleaved DCF reacts with free radical and forms fluorescent adducts. Recently modified versions of the DCFH-DA are carboxymethyl or CM-DCFH-DA which shows a more persistent fluorescence for prolonged duration. It has been frequently observed in the author's lab and other studies that fluorescence levels are readily affected by the type of sample, time of incubation and the instrument used. Therefore, care must be taken while estimating the ROS levels. There are a number of other fluorescent probes available for determination of calcium ions (Fluo-4 AM), mitochondrial superoxide (MitoSox) and mitochondrial membrane potential (JC-1).

B. *TBARS assay*

Thiobarbituric acid reactive species or TBARS is one of the oldest antioxidant assays and also one of the most widely used methods for the detection of lipid oxidation. In this method a lipid peroxidation product called malondialdehyde (MDA) is estimated after a chemical reaction with thiobarbituric acid. Malondialdehyde is the secondary product of ROS which is formed by the oxidation of unsaturated fatty acids. On reacting with thiobarbituric acid (TBA), malondialdehyde forms a pink pigment which is then measured spectrophotometrically at the absorption maximum of 532–535 nm. Various reference and model molecules are also used to set the standards for this assay and for absolute quantification. Wijewickreme has suggested the use of linoleic acid and its emulsions with detergents such as SDS or Tween (Wijewickreme et al. 1997). The use of ethanol in this assay is a debated issue. Studies by Belguendouz et al. (1997) have revealed that the presence or absence of ethanol did not influence the antioxidant activity of the samples. The method was reviewed by Antolovich et al. (2002).

7.2.2 Flow Cytometry-Based Assays

Flow cytometry is a technique that is based on the separation of cells using hydrodynamic focusing and reading of specific molecule using laser excitation and subsequent detection. The advantage of flow cytometry-based assays is that they represent a semiquantitative assessment and also distinguishes between the cells showing differential ROS content, which can never be distinguished using spectrometric techniques. A significantly large number of fluorescent probes are now commercially available which show a binding with specific type of reactive oxygen species (ROS). A list of commercially available probes used for identification reactive oxygen species in flow cytometry is provided in Table 7.1.

Conventionally, reactive oxygen species is determined using DCFH-DA (as described in Sect. 7.2.1). More advanced type of fluorescent probes which are modified version of DCFH-DA is also being used nowadays. Carboxymethyl derivative (CM-DCFH-DA) is much popular choice due to long shelf life and higher quantum yield. Figures 7.2a and 1.2b illustrate the results of flow cytometry-based assays.

7.2.3 Electron Spin Resonance (ESR)-Based Assays

Electron spin resonance or ESR spectrometry is a state-of-the-art technique which has gained attention recently. This technique is powerful enough to specifically detect the free radicals that are involved in autoxidation and cross-oxidation process. As an advantage, the ESR technique is sensitive to several stable free radicals such as di-*tert*-butyl nitroxide (TBN). However, the drawback is that ESR is not able to detect the reactive, short-lived free radicals involved in autoxidation and oxidation of other biomolecules (lifetime varies from 10^{-29} s for the hydroxyl radical to several seconds for the peroxyl radicals). The anomalies of the ESR have been overcome by using several approaches such as continuous flow systems, pulse radiolysis, UV photolysis and spin trapping methods; among these the spin trap method is the most commonly used. Spin trapping involves the use of an additional compound in the reaction called the spin trap, which is capable of reacting with free radicals to fairly stable adducts. These adducts are later detected with the help of ESR scanner.

Some common examples of spin traps which are commercially available include different types of nitroso compounds such as a-phenyl-*tert*-butylnitrone (PBN), *tert*-nitrosobutane (tNB), 5,5-dimethylpyrroline-*N*-oxide (DMPO), a-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) and 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS) (Antolovich et al. 2002).

7.2.4 Microscopy-Based Assays

Using similar fluorescent probes which we discussed in Sect. 7.2.2., we may also prefer to directly visualize the generation and persistence of the reactive oxygen

Table 7.1 Commonly used fluorescent probes used for ROS/RNS detection that are commercially available

Detection reagents (probes)	Reactive oxygen species
RedoxSensor Red CC-1	Hydrogen peroxide
Carboxy-H_2DCFDA	
CM-H_2DCFDA	
Dihydrocalcein AM	
Dihydrorhodamine	
H_2DCFDA	
Lucigenin	
Luminol	
Proxyl fluorescamine	Hydroxyl radical
TEMPO-9-	
3′-(*p*-Aminophenyl) fluorescein (APF)	
3′-(*p*-Hydroxyphenyl) fluorescein (HPF)	
CM-H_2DCFDA	
Luminol	Hypochlorous acid
Aminophenyl fluorescein (APF)	
Dihydrorhodamine	
Luminol	Nitric oxide
DAF-FM	
DAF-FM diacetate	
2,3-Diaminonaphthalene	
Luminol	Peroxyl radical, includes alkylperoxyl and hydroperoxyl radicals (wherein R = H)
cis-Parinaric acid	
RedoxSensor red CC-1	
BODIPY FL EDA	
BODIPY 665/676	
H_2DCFDA	
Carboxy-H_2DCFDA	
CM-H_2DCFDA	
DPPP	
Luminol	Peroxynitrite anion
Dihydrorhodamine	
3′-(*p*-Aminophenyl) fluorescein (APF)	
3′-(*p*-Hydroxyphenyl) fluorescein (HPF)	
H_2DCFDA	
Carboxy-H_2DCFDA	
CM-H_2DCFDA	
Coelenterazine	
Trans-1-(2′-methoxyvinyl)pyrene	Singlet oxygen
Singlet oxygen sensor green reagent	

(continued)

Table 7.1 (continued)

Detection reagents (probes)	Reactive oxygen species
Lucigenin	Superoxide anion
Luminol	
Coelenterazine	
Dihydroethidium	
Fc OxyBURST Green assay reagent	
OxyBURST Green H_2HFF BSA	
XTT, MTT, NBT	
MCLA	
RedoxSensor Red CC-1	
TEMPO-9-AC	

Adapted and redrawn from www.Lifetechnologies/thermofisher.com

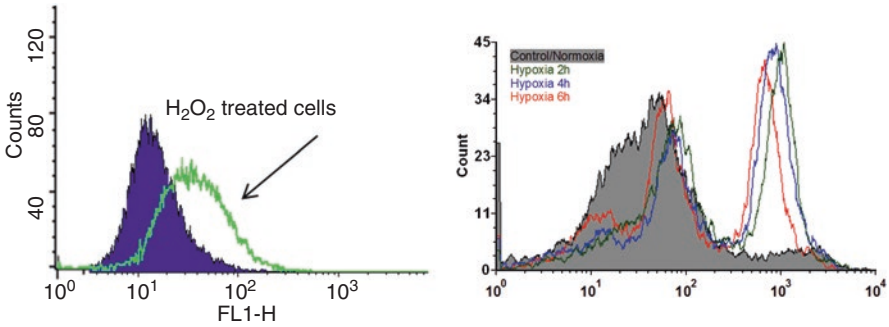

Fig 7.2 Fluorescence histograms of DCFH-DA-stained cells obtained using flow cytometry. (**a**) *Blue-coloured* filled plot represents the fluorescence of control cells, while unfilled *green* histogram represents fluorescence intensity of cells stimulated with hydrogen peroxide (known to induce reactive oxygen species). (**b**) Histogram overlays of various cells stimulated using hydrogen peroxide at different time intervals (*grey-shaded* histogram represents control cells). Note that there are two types of populations in this dataset, some cells which show higher fluorescence (graphs towards extreme *right*), while other populations (towards *left*) show lower fluorescence

species using fluorescent microscopy. This has two advantages; first it can be used to confirm and correlate the results of flow cytometry; second it helps the user to rule out the artefacts.

The most common ROS indicator used is DCFH-DA which shows green fluorescence, and the degree of fluorescence is directly associated with the degree of free radicals generated. Figure 7.2 depicts the difference in the cells under oxidative stress in contrast to normal cells (Fig. 7.3).

Furthermore, a number of dyes (fluorescent probes) are available for the determination of secondary effects of oxidative stress such changes in mitochondrial membrane potential or direct determination of mitochondrial superoxides (MitoSox). The MitoSox-stained cells are shown in Fig 7.4.

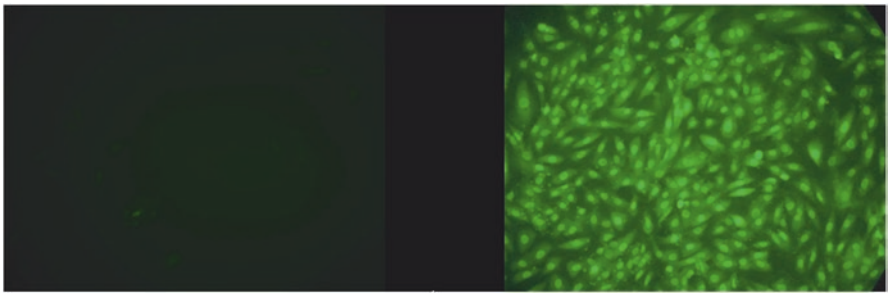

Fig 7.3 Photomicrographs of the cells incubated with DCFH-DA showing relative difference in the fluorescence of cells: (**a**) no oxidative stress or normoxic control and (**b**) cells pre-exposed to hydrogen peroxide

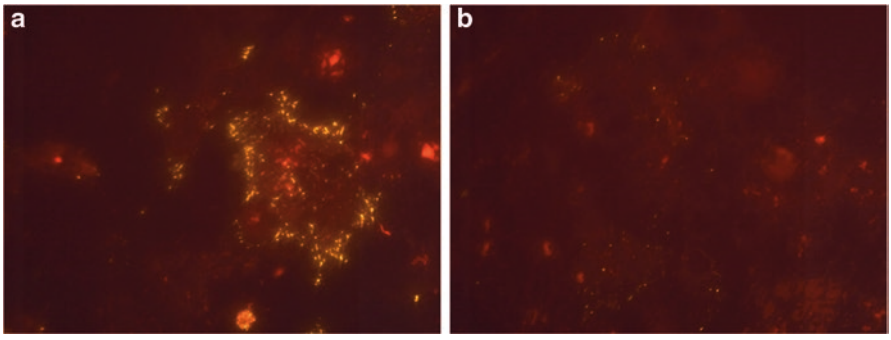

Fig 7.4 Mitochondrial superoxide determination using MitoSox. (**a**) Control cells. (**b**) Cells exposed to simulated oxidative stress

7.3 H9c2 as Preferred Model System

H9c2 (2–1) is basically an incompletely differentiated cell, of cardiac origin, therefore named cardiomyoblast. These were purified and established by B. Kimes and B. Brandt and exhibits many of the properties of the skeletal muscle (Kimes, Brandt, 1976). It is a subclone of the original clonal cell line obtained from embryonic heart tissue of BD1X strain of rat. On terminal differentiation these cells fuse to form multinucleated myotubes and also respond to acetylcholine stimulation showing rhythmic contraction. Fusion occurs faster if the serum concentration in the medium is reduced to 1 %. These cells can be successfully cultured on Dulbecco's Modified Eagle's Medium (DMEM) – a commonly used cell culture media. To make the complete growth medium, DMEM is added with 10% foetal bovine serum, 1% amphotericin and penicillin and streptomycin. CO_2 incubators are used for culturing these cells which maintain of 95% air, 5% carbon dioxide (CO_2) and 37 °C temperature. These cells behave well with the artificial stimulation and different type of oxidative stress simulations. Various studies at the author's lab and many other research labs worldwide have demonstrated the use of H9c2 for oxidative stress-related studies (Fig. 7.5).

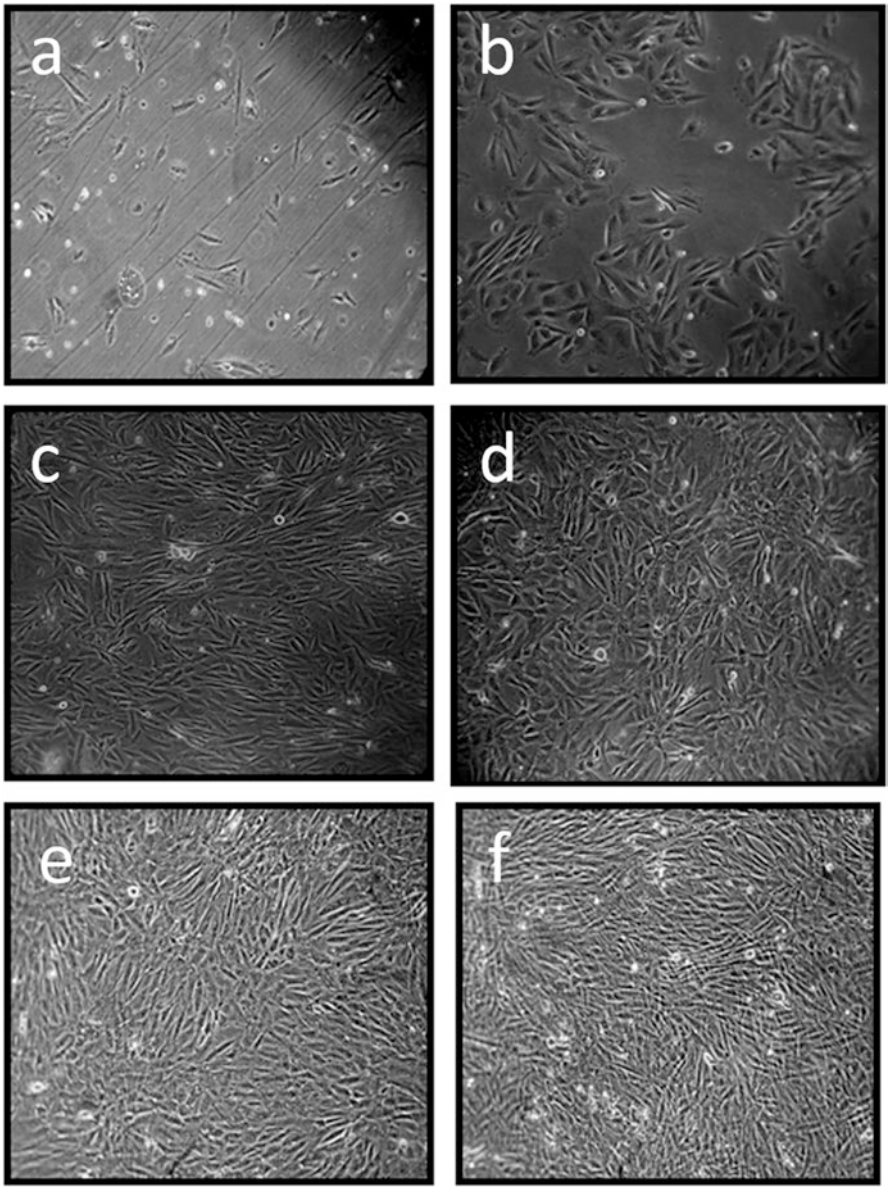

Fig. 7.5 Different stages of growth of H9c2 cardiomyoblasts when grown in DMEM. Phase contrast, 10 X objective. (**a**) Cells after 24 h of seeding start differentiating. (**b**) Rapid division begins after 48 h–72 h. (**c**) Sub-confluence after 72 h. (**d**) Confluence after 4 days. (**e**) Over confluent cells beyond 4 days. (**f**) Cells form overlapping layers if grown beyond 1 week

7.4 Oxidative Stress Biomarkers in Plasma

In most of the cases, for clinical reasons and ethical issues, it is not possible to obtain any type of sample for analysis of oxidative stress and reactive oxygen species (ROS), and therefore some of the non-invasive and semi-invasive techniques are used. Plasma is one of the most preferred samples among biological fluids to determine various pathophysiological conditions. Although aforesaid biochemical methods may be used to analyse the oxidative stress and therefore underlying mechanisms of the oxidative stress induced pathology, but a deeper insight may also be obtained by global proteomics profiling of the plasma samples. Studies at the author's lab have already shown that several proteins that play active role in direct or indirect scavenging of reactive oxygen species are altered during environmental stress. Therefore classical proteomic analysis is also an additional augmentation.

7.5 Future Prospects

Based on the shortcoming of existing methods that they are not highly specific for a particular type of radical and also the life time of the radical is a small fraction of second, it is important to look forward to develop newer methods which could be robust, less time-consuming and cost-effective. Nevertheless, classical methods which predominate the oxidative stress research need to be well understood for their merits and disadvantages before making a choice for experiments. A number of standard methods for detecting the antioxidant potential of the cells that include immunoblotting of the protein involved in antioxidant defence (such as catalase, glutathione peroxidase, thioredoxin reductase, etc.) are often used to determine the status of antioxidant proteins in the cells. Additionally, biological activity using spectrometry-based kinetic methods for antioxidant enzymes or non-enzyme antioxidants such as glutathione is gaining interest. However, there is not any integrated index for overall representation of total oxidative stress level or antioxidant defence of a biological sample. A few labs and commercial manufactures are working on development of such index. Such index may have score for each of the key parameters of oxidative stress and antioxidant defence based on their contribution, ultimately a net value of this index would be a measure to represent the antioxidant potential of the cell or a biological sample. The use of standard antioxidant systems for reference such as Trolox or ascorbate also needs to be revised, and more efficient and sensitive antioxidants must be included in the common antioxidant practice. Cerium oxide nanoparticles have recently emerged as important antioxidants and therefore can be evaluated for their use as reference antioxidants.

Acknowledgements Authors would like to acknowledge Anamika Gangwar, DST-INSPIRE fellow at Defence Institute of Physiology and Allied Sciences (DIPAS, DRDO), who kindly provided the figures of flow cytometry and fluorescence microscopy used in the chapter.

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